**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 6 : | | (11) International Publication Number: |
|---|---|---|
| C12N 15/19, 15/25, 15/26, C12P 21/08, C12N 15/63 | A2 | WO 96/21014 |
| | | (43) International Publication Date: 11 July 1996 (11.07.96) |

(21) International Application Number: PCT/US95/16852

(22) International Filing Date: 22 December 1995 (22.12.95)

(30) Priority Data:
08/367,071 30 December 1994 (30.12.94) US

(71) Applicant: CHIRON VIAGENE, INC. [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).

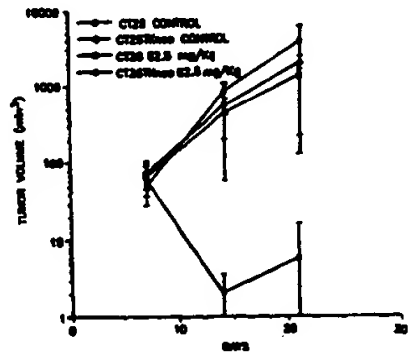
(72) Inventors: JOLLY, Douglas, J.; 277 Hillcrest Drive, Leucadia, CA 92024 (US). BARBER, Jack, R.; 11168 Carlotta Street, San Diego, CA 92129 (US). CHANG, Stephen, M., W.; 12912 Camino del Valle, Poway, CA 92064 (US). RESPESS, James, G.; 4966 Lamont Street, San Diego, CA 92109 (US). ALLEN, John, R.; 1907 Elm Ridge Drive, Vista Ridge, CA 92083 (US). BODNER, Mordechai; 3480 Juniper Street, San Diego, CA 92104 (US). CHONG, Kimberly; 14955 Avenue Venusto #7, San Diego, CA 92128 (US). DE LA VEGA, Dan, Jr.; 3243 Grim Avenue, San Diego, CA 92104 (US). DePOLO, Nicholas, J.; 904 Santa Estrella, Solana Beach, CA 92605 (US). HSU, David, Chi-Tang; 8012 Camino Tranquilo, San Diego, CA 92122 (US). IBANEZ, Carlos, E.; 13592 Millpond Way, San Diego, CA 92129 (US). MITTELSTAEDT, Denice, M.; 13687 Quinton Road, San Diego, CA 92129 (US). PRUSSAK, Charles, E.; 5121 Camino Playa Malaya, San Diego, CA 92124 (US).

(74) Agents: KRUSE, Norman, J. et al.; Chiron Corporation, Intellectual Property - R440, P.O. Box 8097, Emeryville, CA 94662-8097 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

**Published**
*Without international search report and to be republished upon receipt of that report.*

(54) Title: PRODUCTION AND ADMINISTRATION OF HIGH TITER RECOMBINANT RETROVIRUSES



(57) Abstract

Methods are provided for obtaining measurable levels of a protein, nucleic acid molecule, or enzymatic product in a bodily fluid of cells of a human, comprising the step of administering human a recombinant retroviral preparation having a titer on HT1080 cells of greater than $10^5$ cfu/ml, wherein the recombinant retroviral preparation is capable of directing the expression of a protein, nucleic acid molecule, or enzyme which generates an enzymatic product, such that measurable levels of the protein, nucleic acid molecule, or enzymatic product may be obtained in the bodily fluid of cells of the human.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|---|---|---|---|---|---|
| **AM** | Armenia | **GB** | United Kingdom | **MW** | Malawi |
| **AT** | Austria | **GE** | Georgia | **MX** | Mexico |
| **AU** | Australia | **GN** | Guinea | **NE** | Niger |
| **BB** | Barbados | **GR** | Greece | **NL** | Netherlands |
| **BE** | Belgium | **HU** | Hungary | **NO** | Norway |
| **BF** | Burkina Faso | **IE** | Ireland | **NZ** | New Zealand |
| **BG** | Bulgaria | **IT** | Italy | **PL** | Poland |
| **BJ** | Benin | **JP** | Japan | **PT** | Portugal |
| **BR** | Brazil | **KE** | Kenya | **RO** | Romania |
| **BY** | Belarus | **KG** | Kyrgystan | **RU** | Russian Federation |
| **CA** | Canada | **KP** | Democratic People's Republic of Korea | **SD** | Sudan |
| **CF** | Central African Republic | | | **SE** | Sweden |
| **CG** | Congo | **KR** | Republic of Korea | **SG** | Singapore |
| **CH** | Switzerland | **KZ** | Kazakhstan | **SI** | Slovenia |
| **CI** | Côte d'Ivoire | **LI** | Liechtenstein | **SK** | Slovakia |
| **CM** | Cameroon | **LK** | Sri Lanka | **SN** | Senegal |
| **CN** | China | **LR** | Liberia | **SZ** | Swaziland |
| **CS** | Czechoslovakia | **LT** | Lithuania | **TD** | Chad |
| **CZ** | Czech Republic | **LU** | Luxembourg | **TG** | Togo |
| **DE** | Germany | **LV** | Latvia | **TJ** | Tajikistan |
| **DK** | Denmark | **MC** | Monaco | **TT** | Trinidad and Tobago |
| **EE** | Estonia | **MD** | Republic of Moldova | **UA** | Ukraine |
| **ES** | Spain | **MG** | Madagascar | **UG** | Uganda |
| **FI** | Finland | **ML** | Mali | **US** | United States of America |
| **FR** | France | **MN** | Mongolia | **UZ** | Uzbekistan |
| **GA** | Gabon | **MR** | Mauritania | **VN** | Viet Nam |

# PRODUCTION AND ADMINISTRATION OF HIGH TITER RECOMBINANT RETROVIRUSES

## Description

The present invention relates generally to .recombinant retroviruses, and more specifically, to high titer recombinant retroviral particle preparations suitable for a variety of applications.

## Background Of The Invention

Since the discovery of DNA in the 1940s and continuing through the most recent era of recombinant DNA technology, substantial research has been undertaken in order to realize the possibility that the course of disease may be affected through interaction with the nucleic acids of living organisms. Most recently, a wide variety of methods have been described for altering or affecting genes, including for example, viral vectors derived from retroviruses, adenoviruses, vaccinia viruses, herpes viruses, and adeno-associated viruses (see Jolly, *Cancer Gene Therapy 1*(1):51-64, 1994), as well as direct transfer techniques such as lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature 352*:815-818, 1991), microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991), liposomes of several types (*see, e.g.*, Wang et al., *PNAS 84*:7851-7855, 1987) and administration of nucleic acids alone (WO 90/11092).

Of these techniques, recombinant retroviral gene delivery methods have been most extensively utilized, in part due to: (1) the efficient entry of genetic material (the vector genome) into cells; (2) an active, efficient process of entry into the target cell nucleus; (3) relatively high levels of gene expression; (4) the potential to target particular cellular subtypes through control of the vector-target cell binding and the tissue-specific control of gene expression; (5) a general lack of pre-existing host immunity; and (6) substantial knowledge and clinical experience which has been gained with such vectors.

Briefly, retroviruses are diploid positive-strand RNA viruses that replicate through an integrated DNA intermediate. In particular, upon infection by the RNA virus, the retroviral genome is reverse-transcribed into DNA by a virally encoded reverse transcriptase that is carried as a protein in each retrovirus. The viral DNA is then integrated pseudo-randomly into the host cell genome of the infecting cell, forming a "provirus" which is inherited by daughter cells.

Wild-type retroviral genomes (and their proviral copies) contain three genes (the *gag*, *pol* and *env* genes), which are preceded by a packaging signal ($\psi$), and two long terminal repeat (LTR) sequences which flank both ends. Briefly, the *gag* gene encodes the

internal structural (nucleocapsid) proteins. The *pol* gene codes for the RNA-dependent DNA polymerase which reverse transcribes the RNA genome, and the *env* gene encodes the retroviral envelope glycoproteins. The 5' and 3' LTRs contain *cis*-acting elements necessary to promote transcription and polyadenylation of retroviral RNA.

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of retroviral RNA into particles (the ψ sequence). Removal of the packaging signal prevents encapsidation (packaging of retroviral RNA into infectious virions) of genomic RNA, although the resulting mutant can still direct synthesis of all proteins encoded in the viral genome.

Recombinant retroviruses and various uses thereof have been described in numerous references including, for example, Mann et al. (*Cell 33:*153, 1983), Cane and Mulligan (*Proc. Nat'l. Acad. Sci. USA 81*:6349, 1984), Miller et al., *Human Gene Therapy* 1:5-14, 1990, U.S. Patent Nos. 4,405,712; 4,861,719; 4,980,289 and PCT Application Nos. WO 89/02,468; WO 89/05,349 and WO 90/02,806. Briefly, a foreign gene of interest may be incorporated into the retrovirus in place of the normal retroviral RNA. When the retrovirus injects its RNA into a cell, the foreign gene is also introduced into the cell, and may then be integrated into the host's cellular DNA as if it were the retrovirus itself. Expression of this foreign gene within the host results in expression of the foreign protein by the host cell.

One disadvantage, however, of recombinant retroviruses is that they principally infect only replicating cells, thereby making efficient direct gene transfer difficult. Indeed, some scientists have suggested that other, more efficient methods of gene transfer, such as direct administration of pure plasmid DNA, be utilized (Davis et al., *Human Gene Therapy 4*:733-740, 1993).

In order to increase the efficacy of recombinant retroviruses, methods which have been suggested for increasing the efficacy of recombinant retroviruses have principally been aimed at inducing the desired target cells to replicate, thereby allowing the retroviruses to infect the cells. Such methods have included, for example chemical treatment with 10% carbon tetrachloride in mineral oil (Kaleko et al., *Human Gene Therapy 2*:27-32, 1991). Alternatively, others have suggested excising large portions of the liver (e.g., 70% in Rettinger et al., *PNAS 91*:1460-1464, 1994; 70% in Moscioni et al., *Surgery 113*:304-311, 1993; ) in order to stimulate the rapid division of hepatocytes and thereby increase the infection of such cells.

One further disadvantage of recombinant retroviruses, is that serum from primates (*e.g.*, humans) is known to cause inactivation by an antibody independent complement lysis method. In particular retroviruses of avian, murine, feline, and simian origin are all inactivated and lysed by normal human serum. (Welsh et al., *Nature 257*:612-

614, 1975; Welsh et al., *Virology 74*:432-440, 1976; Banapour et al., *Virology 152*:268-271, 1986; and Cooper et al., Immunology of the Complement System, Pub., American Press, Inc., pp. 139-162, 1986) The scientific literature has also reported that replication competent murine amphotropic retroviruses injected intravenously into primates are cleared within 15 minutes and that the disappearance is mediated, wholly or in part, by primate complement. (Cornetta et al., *Human Gene Therapy 2*:5-14, 1991; Cornetta et al., *Human Gene Therapy 1*:15-30, 1990; Banapour et al., *Virology 152*:268-271, 1986)

In order to increase the affect of recombinant retroviruses that are delivered *in vivo*, the present invention provides recombinant retrovirus compositions which are capable of surviving inactivation in human serum. In addition, the present invention provides high titer recombinant retrovirus compositions which allow delivery of therapeutics or palliatives by routes not previously deemed possible, and without the need to induce replication of cells by chemical treatment or by excision of a target organ such as the liver. The present invention provides these, as well as other related advantages.

## Summary of the Invention

Briefly stated, the present invention provides high titer compositions comprising recombinant retroviruses; as well as methods for utilizing these compositions. Within one aspect of the present invention, methods are provided for obtaining measurable levels of a protein, nucleic acid molecule, or enzymatic product in a bodily fluid or cells of a human, comprising the step of administering to a human a recombinant retroviral preparation having a titer on HT1080 cells of greater than $10^5$ cfu/ml, wherein the recombinant retroviral preparation is capable of directing the expression of a protein, nucleic acid molecule, or enzyme which generates an enzymatic product, such that measurable levels of the protein, nucleic acid molecule, or enzymatic product may be obtained in the bodily fluid or cells of said human. Within certain embodiments, the titer may be greater than $10^6$ cfu/ml, $10^7$ cfu/ml, $10^8$ cfu/ml, $10^9$ cfu/ml, $10^{10}$ cfu/ml, or $10^{11}$ cfu/ml.

Within other aspects of the invention, methods are provided for obtaining measurable levels of a protein, nucleic acid molecule, or enzymatic product in a bodily fluid or cells of a human, comprising the steps of administering to a human a recombinant retroviral preparation having a titer in human serum and on HT1080 cells equivalent to its' titer in heat-inactivated serum and on HT1080 cells, wherein the recombinant retroviral preparation is capable of directing the expression of a protein, nucleic acid molecule, or enzyme which generates an enzymatic product, such that measurable levels of the protein, nucleic acid molecule, or enzymatic product may be obtained in the bodily fluid or cells of said human.

As utilized within the context of the present invention, "measurable levels" of a protein, nucleic acid molecule, or enzymatic product refers to a statisically significant level of detection over background, utilizing any suitable technique (e.g., antibody-mediated detection of a protein, PCR analysis for the presence of a nucleic acid molecule, or visualization of enzymatic products). Further, as utilized within the context of the present invention, "equivalent" titers are deemed to be those which are substantially the same within a given assay, generally, within about three-fold of each other.

Within certain embodiments of the invention, the recombinant retrovirus is administered to a site such as the cerebral spinal fluid, bone marrow, joints, arterial endothelial cells, rectum, buccal/sublingual, vagina, the lymph system, to an organ selected from the group consisting of lung, liver, spleen, skin, blood and brain, or to a site selected from the group consisting of tumors and interstitial spaces. Within other embodiments, the recombinant retrovirus may be administered intraocularly, intranasally, sublinually, orally, topically, intravesically, intrathecally, topically, intravenously, intraperitoneally, intracranially, intramuscularly, or subcutaneously.

Within yet other embodiments of the present invention, the protein is a viral antigen obtained from a virus such as influenza virus, respiratory syncytial virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hantavirus, HTLV I, HTLV II and CMV. Within other embodiments, the protein is a cytokine such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, γ-IFN, G-CSF and GM-CSF, or a receptor for any of these cytokines.

Within another embodiment, the nucleic acid molecule may be an antisense sequence, a non-coding non-heterologous sense sequence, and a ribozyme sequence. Within yet another aspect, the protein is a toxin.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety as if each were individually noted for incorporation.

Brief Description of the Drawings

Figure 1 is a schematic illustration of p31N2R5(+).

Figure 2 is a schematic illustration of pN2R3(-).

Figure 3 is a schematic illustration of p31N25Δ(+).

Figure 4 is a schematic illustration of pN2R3(+).

Figure 5 is a schematic illustration of pN2R5(-).

Figure 6 is a schematic illustration of p31N25Δ(+).

Figure 7 is a schematic illustration of pTKΔA.

Figure 8 is a schematic illustration of pPrTKΔA.

Figure 9 is a schematic illustration of pTK-1 and pTK-3.

Figure 10 is a bar graph which illustrates the effect of Ganciclovir on CT26, CT26 bgal and CT26TK Neo cells.

Figure 11 is a graph which illustrates the effect of tumor volume over time in a Ganciclovir dose study of mice injected with CT26TK Neo.

Figure 12 is a series of four photographs of mice, illustrating the effect of different dose regimens of Ganciclovir on intraperitoneal tumor growth.

Figure 13 is a series of four photographs of mice, illustrating the effect of different dose regimens of Ganciclovir on subcutaneous tumor growth.

Figure 14 is a graph illustrating the effect of Ganciclovir in CT26 versus CT26TK Neo cells.

Figure 15 is a graph demonstrating retention of viral activity upon reconstitution of a representative recombinant retrovirus lyophilized in a formulation buffer containing mannitol.

Figure 16 is a graph demonstrating retention of viral activity upon reconstitution of a representative recombinant retrovirus lyophilized in a formulation buffer containing lactose.

Figure 17 is a graph demonstrating retention of viral activity upon reconstitution of a representative recombinant retrovirus lyophilized in a formulation buffer containing trehalose.

Figures 18A-18D are representative graphs comparing stability of liquid non-lyophilized recombinant retrovirus stored at -80°C versus lyophilized formulated recombinant retrovirus stored at -20°C, using various saccharides.

Figure 19 is a bar graph which depicts the results of a reverse transcriptase assay on samples sliced from a gel. Slice 1 is from the lowest part of the gel, and slice 8 from the highest.

Figure 20 depicts a 8% to 25% gradient polyacrylamide gel. Lane 1 is DA/β gal S-500 purified; Lane 2 is DA/βgal crude supernatant; Lane 3 is HIV-IT crude supernatant; lane 4 is DAC 6A3 crude supernatant; Lane 5 is HBc/SA2 crude supernatant; and Lane 5 is molecular weight markers.

Figure 21 is a schematic illustration of pDHF811.

Figures 22A-D are graphs which indicate the total quantity of lactate in low seed and high seed cultures (Figures 22A and 22B, respectively) and the level of lactate production per day in low seed and high seed cultures (Figures 22C and 22D, respectively).

Figure 23 is a bar graph which depicts the titer of the cell line 2X-ß-gal under different initial seeding conditions.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

"Vector construct", "retroviral vector", "recombinant vector", and "recombinant retroviral vector" refers to a nucleic acid construct which carries, and within certain embodiments, is capable of directing the expression of a nucleic acid molecule of interest. The retroviral vector must include at least one transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by other means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Such vector constructs must also include a packaging signal, long terminal repeats (LTRs) or portion thereof, and positive and negative strand primer binding sites appropriate to the retrovirus used (if these are not already present in the retroviral vector). Optionally, the recombinant retroviral vector may also include a signal which directs polyadenylation, selectable markers such as Neomycin resistance, TK, hygromycin resistance, phleomycin resistinacne histidinol resistance, or DHFR, as well as one or more restriction sites and a translation termination sequence. By way of example, such vectors typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis, and a 3' LTR or a portion thereof.

"Recombinant retrovirus", 'retroviral gene delivery vehicle" and 'retroviral vector particle" as utilized within the present invention refers to a retrovirus which carries at least one gene of interest. The retrovirus may also contain a selectable marker. The recombinant retrovirus is capable of reverse transcribing its genetic material into DNA and incorporating this genetic material into a host cell's DNA upon infection.

As noted above, the present invention provides high titer recombinant retroviral preparations which are suitable for administration to humans. Such preparations provide the unexpected result of providing efficatious gene therapy for a variety of diseases (and by a variety of routes) that were previously not amenable for gene therapy. In addition, the present invention provides recombinant retroviruses which are capable of surviving inactivation in human serum, thereby allowing more efficient gene transfer over prolonged periods of time.

A. PREPARATION OF RECOMBINANT RETROVIRAL VECTORS, PACKAGING CELLS, PRODUCER CELLS AND RECOMBINANT RETROVIRUSES

As noted above, the present invention provides recombinant retroviruses which are constructed to carry or express a selected nucleic acid molecule of interest. Briefly, numerous retroviral gene delivery vehicles may be utilized within the context of the present invention, including for example those described in EP 0,415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 9311230; WO 9310218; Vile and Hart, *Cancer Res. 53*:3860-3864, 1993; Vile and Hart, *Cancer Res. 53*:962-967, 1993; Ram et al., *Cancer Res. 53*:83-88, 1993; Takamiya et al., *J. Neurosci. Res. 33*:493-503, 1992; Baba et al., *J. Neurosurg. 79*:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO 91/02805). Particularly preferred recombinant retroviruses include those described in WO 91/02805.

Retroviral gene delivery vehicles of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (see RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Preferred retroviruses for the preparation or construction of retroviral gene delivery vehicles of the present invention include retroviruses selected from the group consisting of Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe, *J. Virol. 19*:19-25, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Maryland), or isolated from known sources using commonly available techniques.

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral gene delivery vehicles given the disclosure provided herein, and standard recombinant techniques (*e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS 82*:488, 1985). In addition, within certain embodiments of the invention, portions of the retroviral gene delivery vehicles may be derived from different retroviruses. For example, within one embodiment of the invention, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

Within preferred aspects of the present invention, recombinant retroviruses may be made by introducing a vector construct as discussed above, into a cell (termed a "packaging cell") which contains those elements necessary for production of infectious

recombinant retrovirus which are lacking in the vector construct. A wide variety of retrovector constructs may be utilized within the present invention in order to prepare recombinant retroviruses. For example, within one aspect of the present invention retrovector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks gag/pol or env coding sequences. Briefly, Long Terminal Repeats ("LTRs") are subdivided into three elements, designated U5, R and U3. These elements contain a variety of signals which are responsible for the biological activity of a retrovirus, including for example, promoter and enhancer elements which are located within U3. LTRs may be readily identified in the provirus due to their precise duplication at either end of the genome. As utilized herein, a 5' LTR should be understood to include a 5' promoter element and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector. The 3' LTR should be understood to include a polyadenylation signal, and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector.

The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of skill in the art. For example, retroviral tRNA binds to a tRNA binding site by Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR.

In addition to a 5' and 3' LTR, tRNA binding site, and origin of second strand DNA synthesis, certain preferred retrovector constructs which are provided herein also comprise a packaging signal, as well as one or more nucleic acid molecules (*e.g.*, heterologous sequences), each of which is discussed in more detail below.

Within one aspect of the invention, retrovector constructs are provided which lack both *gag/pol* and *env* coding sequences. As utilized herein, the phrase "lacks *gag/pol* or *env* coding sequences" should be understood to mean that the retrovector does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably at least 8 consecutive nucleotides which are found in *gag/pol* or *env* genes, and in particular, within *gag/pol* or *env* expression cassettes that are used to construct packaging cell lines for the retrovector construct.

Packaging cell lines suitable for use with the above-described retrovector constructs may be readily prepared (see U.S. Serial No. 08/240,030, filed May 9, 1994; see also

WO 92/05266), and utilized to create producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant vector particles. Within particularly preferred embodiments of the present invention packaging cell lines are made from human (*e.g.*, HT1080 cells) or mink parent cell lines, thereby allowing production of recombinant retroviruses that are capable of surviving inactivation in human serum. One representative assay that may be utilized in order to determine the whether recombinant retroviruses are inactivated in human serum is set forth in more detail below in Example 11.

Utilizing the methods of the present invention as disclosed herein, packaging cell lines that produce greater than recombinant retroviral particles at titers greater than $10^6$ or $10^7$ cfu/ml (in crude supernatant) may readily be obtained. In addition, it should be noted that such titers are generally obtained from titer assays on HT1080 cells, which produce a three-fold lower titer than titers obtained on murine 3T3 cells.

## B. RECOMBINANT RETROVIRUSES WHICH CARRY AND/OR EXPRESS A DESIRED NUCLEIC ACID MOLECULE

A wide variety of nucleic acid molecules may be carried and/or expressed by the recombinant retroviruses of the present invention. Generally, the nucleic acid molecules which are described herein do not occur naturally in the recombinant retrovirus that carries it, and provides some desirable benefit, typically an ability to fight a disease, or other pathogenic agent or condition. As used herein, "pathogenic agent" refers to a cell that is responsible for a disease state. Representative examples of pathogenic agents include tumor cells, autoreactive immune cells, hormone secreting cells, cells which lack a function that they would normally have, cells that have an additional inappropriate gene expression which does not normally occur in that cell type, and cells infected with bacteria, viruses, or other intracellular parasites. In addition, as used herein "pathogenic agent" may also refer to a cell that over-expresses or inappropriately expresses a recombinant retrovirus (*e.g.*, in the wrong cell type), or that has become tumorigenic due to inappropriate insertion into a host cell's genome.

Examples of nucleic acid molecules which may be carried and/or expressed by the recombinant retroviruses of the present invention include genes and other nucleic acid molecules which encode a substance, as well as biologically active nucleic acid molecules such as inactivating sequences that incorporate into a specified intracellular nucleic acid molecule and inactivate that molecule. A nucleic acid molecule is considered to be biologically active when the molecule itself provides the desired benefit without requiring the expression of a substance. For example, the biologically active nucleic acid molecule may be an inactivating sequence that incorporates into a specified intracellular nucleic acid molecule

and inactivates that molecule, or the molecule may be a tRNA, rRNA or mRNA that has a configuration that provides a binding capability.

Substances which may be encoded by the nucleic acid molecules described herein include proteins (*e.g.*, antibodies including single chain molecules), immunostimulatory molecules (such as antigens) immunosuppressive molecules, blocking agents, palliatives (such as toxins, antisense ribonucleic acids, ribozymes, enzymes, and other material capable of inhibiting a function of a pathogenic agent) cytokines, various polypeptides or peptide hormones, their agonists or antagonists, where these hormones can be derived from tissues such as the pituitary, hypothalamus, kidney, endothelial cells, liver, pancreas, bone, hemopoetic marrow, and adrenal. Such polypeptides can be used for induction of growth, regression of tissue, suppression of immune responses, apoptosis, gene expression, blocking receptor-ligand interaction, immune responses and can be treatment for certain anemias, diabetes, infections, high blood pressure, abnormal blood chemistry or chemistries (*e.g.*, elevated blood cholesterol, deficiency of blood clotting factors, elevated LDL with lowered HDL), levels of Alzheimer associated amaloid protein, bone erosion/calcium deposition, and controlling levels of various metabolites such as steroid hormones, purines, and pyrimidines.

For palliatives, when "capable of inhibiting a function" is utilized within the context of the present invention, it should be understood that the palliative either directly inhibits the function or indirectly does so, for example, by converting an agent present in the cells from one which would not normally inhibit a function of the pathogenic agent to one which does. Examples of such functions for viral diseases include adsorption, replication, gene expression, assembly, and exit of the virus from infected cells. Examples of such functions for cancerous diseases include cell replication, susceptibility to external signals (*e.g.*, contact inhibition), and lack of production of anti-oncogene proteins. Examples of such functions for cardiovascular disease include inappropriate growth or accumulation of material in blood vessels, high blood pressure, undesirable blood levels of factors such as cholesterol or low density lipoprotein that predispose to disease, localized hypoxia, and inappropriately high and tissue-damaging levels of free radicals. Examples of such functions for neurological conditions include pain, lack of dopamine production, inability to replace damaged cells, deficiencies in motor control of physical activity, inappropriately low levels of various peptide hormones derived from neurological tissue such as the pituitary or hypothalamus, accumulation of Alzheimer's Disease associated amyloid plaque protein, and inability to regenerate damaged nerve junctions. Examples of such functions for autoimmune or inlammatory disease include inappropriate production of cytokines and lymphokines, inappropriate production and existence of autoimmune antibodies and cellular immune responses, inappropriate disruption of tissues by proteases and collagenases, inhibition of the

normal action of proteases, lack of production of factors normally supplied by destroyed cells, and excessive or aberrant regrowth of tissues under autoimmune attack.

Within one aspect of the present invention, methods are provided for administration of a recombinant retrovirus which directs the expression of a palliative. Within various embodiments, the palliative may be a DNA molecule, an RNA molecule, or some combination of the two.

Representative examples of palliatives that act directly to inhibit the growth of cells include toxins such as ricin (Lamb et al., *Eur. J. Biochem. 148*:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem. 198*:723-732, 1991; Evensen et al., *J. of Biol. Chem. 266*:6848-6852, 1991; Collins et al., *J. of Biol. Chem. 265*:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc. 309*:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem. 260*:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature 306*:551-557, 1983; Sanchez & Holmgren, *PNAS 86*:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem. 255*:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther. 21*:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J. 203*:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys. 200*:418-425, 1980; Irvin, *Arch. Biochem. & Biophys. 169*:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS 84*:4364-4368, 1987; Jackson et al., *Microb. Path. 2*:147-153, 1987), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem. 262*:8707-8711, 1987). A detailed description of recombinant retroviruses which express Russel's Viper Venom is provided in U.S. Serial No. ____, filed December 30, 1994 [Attorney's Docket No. 930049.440].

Within other aspects of the invention, the recombinant retrovirus carries a gene specifying a product which is not in itself toxic, but when processed or modified by a protein, such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, the recombinant retrovirus could carry a gene encoding a proprotein chain, which becomes toxic upon processing by the HIV protease. More specifically, a synthetic inactive proprotein form of the toxic ricin or diphtheria A chains could be cleaved to the active form by arranging for the HIV virally encoded protease to recognize and cleave off an appropriate "pro" element.

Within yet another aspect of the invention, the recombinant retrovirus directs the expression of a substance capable of activating an otherwise inactive precursor into an active inhibitor of a pathogenic agent, or a conditional toxic palliative, which are palliatives that are toxic for the cell expressing the pathogenic condition. As should be evident given the disclosure provided herein, a wide variety of inactive precursors may be converted into active inhibitors of a pathogenic agent. For example, antiviral nucleoside analogues such as AZT or ddI are metabolized by cellular mechanisms to the nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase, and thus viral replication

(Furmam et al., *Proc. Natl. Acad. Sci. USA 83*:8333-8337, 1986). Recombinant retroviruses which direct the expression of a gene product (*e.g.*, a protein) such as Herpes Simplex Virus Thymidine Kinase (HSVTK) or Varicella Zoster Virus Thymidine Kinase (VZVTK) which assists in metabolizing antiviral nucleoside analogues to their active form are therefore useful in activating nucleoside analogue precursors (*e.g.*, AZT or ddC) into their active form. AZT or ddI therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, will be made more efficacious.

Within one embodiment of the invention, the HSVTK gene may be expressed under the control of a constitutive macrophage or T-cell-specific promoter, and introduced into macrophage or T-cells. Constitutive expression of HSVTK results in more effective metabolism of nucleotide analogues such as AZT or ddI to their biologically active nucleotide triphosphate form, and thereby provides greater efficacy, delivery of lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, may also be utilized within the context of the present invention.

Within a related aspect of the present invention, recombinant retroviruses are provided which direct the expression of a substance that activates another compound with little or no cytotoxicity into a toxic product in the presence of a pathogenic agent, thereby effecting localized therapy to the pathogenic agent. In this case, expression of the gene product from the recombinant retrovirus is limited to situations wherein an entity associated with the pathogenic agent, such as an intracellular signal identifying the pathogenic state, is present, thereby avoiding destruction of nonpathogenic cells. This cell-type specificity may also be conferred at the level of infection, by targeting the recombinant retrovirus to cells having or being susceptible to the pathogenic condition.

Within a related aspect of the present invention, recombinant retroviruses are provided which direct the expression of a gene product(s) that activates a compound with little or no cytotoxicity into a toxic product. Briefly, a wide variety of gene products which either directly or indirectly activate a compound with little or no cytotoxicity into a toxic product may be utilized within the context of the present invention. Representative examples of such gene products include HSVTK and VZVTK which selectively monophosphorylate certain purine arabinosides and substituted pyrimidine compounds, converting them to cytotoxic or cytostatic metabolites. More specifically, exposure of the drugs gangicylovir,

acyclovir, or any of their analogues (*e.g.*, FIAC, DHPG) to HSVTK, phosphorylates the drug into its corresponding active nucleotide triphosphate form.

For example, within one embodiment of the invention, the recombinant retrovirus directs the expression of the herpes simplex virus thymidine kinase ("HSVTK") gene downstream, and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Briefly, expression of the tat gene product in human cells infected with HIV and carrying the recombinant retrovirus causes increased production of HSVTK. The cells (either *in vitro* or *in vivo*) are then exposed to a drug such as ganciclovir, acyclovir or its analogues (FIAC, DHPG). As noted above, these drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms. Acyclovir triphosphates inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (see Borrelli et al., *Proc. Natl. Acad. Sci. USA 85*:7572, 1988). Those cells containing the recombinant retrovirus and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs.

Within one embodiment of the invention, expression of a conditionally lethal HSVTK gene may be made even more HIV-specific by including cis-acting elements in the transcript ("CRS/CAR"), which require an additional HIV gene product, rev, for optimal activity (Rosen et al., *Proc. Natl. Acad. Sci. USA 85*:2071, 1988). More generally, cis elements present in mRNAs have been shown in some cases to regulate mRNA stability or translatability. Sequences of this type (*i.e.*, post-transcriptional regulation of gene expression) may be used for event- or tissue-specific regulation of vector gene expression. In addition, multimerization of these sequences (*i.e.*, rev-responsive "CRS/CAR" or tat-responsive "TAR" elements for HIV) may be utilized in order to generate even greater specificity.

In a manner similar to the preceding embodiment, recombinant retroviruses may be generated which carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. Such genes may have no equivalent in mammalian cells, and might come from organisms such as a virus, bacterium, fungus, or protozoan. Representative examples include: *E. coli* guanine phosphoribosyl transferase ("gpt") gene product, which converts thioxanthine into thioxanthine monophosphate (see Besnard et al., *Mol. Cell. Biol. 7*:4139-4141, 1987); alkaline phosphatase, which will convert inactive phosphorylated compounds such as mitomycin phosphate and doxorubicin-phosphate to toxic dephosphorylated compounds; fungal (*e.g.*, *Fusarium oxysporum*) or bacterial cytosine deaminase which will convert 5-fluorocytosine to the toxic compound 5-fluorouracil (Mullen, *PNAS 89*:33, 1992); carboxypeptidase G2 which will cleave the glutamic acid from para-N-bis (2-chloroethyl) aminobenzoyl glutamic acid,

thereby creating a toxic benzoic acid mustard; and Penicillin-V amidase, which will convert phenoxyacetabide derivatives of doxorubicin and melphalan to toxic compounds. Conditionally lethal gene products of this type have application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a nontoxic drug, which is not a purine or pyrimidine analogue, to a cytotoxic form (see Searle et al., *Brit. J. Cancer 53*:377-384, 1986).

Additionally, in the instance where the target pathogen is a mammalian virus, recombinant retroviruses vectors may be constructed to take advantage of the fact that mammalian viruses in general tend to have "immediate early" genes, which are necessary for subsequent transcriptional activation of other viral promoter elements. Gene products of this nature are excellent candidates for intracellular signals (or "identifying agents") of viral infection. Thus, conditionally lethal genes transcribed from transcriptional promoter elements that are responsive to such viral "immediate early" gene products could specifically kill cells infected with any particular virus. Additionally, since the human $\alpha$ and $\beta$ interferon promoter elements are transcriptionally activated in response to infection by a wide variety of nonrelated viruses, the introduction of vectors expressing a conditionally lethal gene product like HSVTK, for example, from these viral-responsive elements (VREs) could result in the destruction of cells infected with a variety of different viruses.

In another embodiment of the invention, recombinant retroviruses are provided that produce substances such as inhibitor palliatives, that inhibit viral assembly. In this context, the recombinant retrovirus codes for defective *gag*, *pol*, *env* or other viral particle proteins or peptides which inhibit in a dominant fashion the assembly of viral particles. Such inhibition occurs because the interaction of normal subunits of the viral particle is disturbed by interaction with the defective subunits.

One way of increasing the effectiveness of inhibitory palliatives is to express inhibitory genes, such as viral inhibitory genes, in conjunction with the expression of genes which increase the probability of infection of the resistant cell by the virus in question. The result is a nonproductive "dead-end" event which would compete for productive infection events. In the specific case of HIV, a recombinant retrovirus may be administered that inhibits HIV replication (by expressing anti-sense tat, etc., as described above) and also overexpress proteins required for infection, such as CD4. In this way, a relatively small number of vector-infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells.

In another embodiment of the invention, recombinant retroviruses are provided for the expression substances such as inhibiting peptides or proteins specific for viral protease. Viral protease cleaves the viral *gag* and *gag/pol* proteins into a number of

smaller peptides. Failure of this cleavage in all cases leads to complete inhibition of production of infectious retroviral particles. The HIV protease is known to be an aspartyl protease, and these are known to be inhibited by peptides made from amino acids from protein or analogues. Recombinant retroviruses that inhibit HIV will express one or multiple fused copies of such peptide inhibitors.

Administration of the recombinant retroviruses discussed above should be effective against many virally linked diseases, cancers, or other pathogenic agents.

Within still other embodiments of the invention, recombinant retroviruses are provided that express a palliative, wherein the palliative has a membrane anchor and acts as an anti-tumor agent(s). Such a palliative may be constructed, for example, as an anti-tumor agent - membrane anchor fusion protein. Briefly, the membrane anchor aspect of the fusion protein may be selected from a variety of sequences, including, for example, the transmembrane domain of well known molecules. Generally, membrane anchor sequences are regions of a protein that bind the protein to a membrane. Customarily, there are two types of anchor sequences that attach a protein to the outer surface of a cell membrane: (1) transmembrane regions that span the lipid bilayer of the cell membrane, and interact with the hydrophobic center region (proteins containing such regions are referred to integral membrane proteins), and (2) domains which interact with an integral membrane protein or with the polar surface of the membrane (such proteins are referred to as peripheral, or extrinsic, proteins).

Membrane anchors for use within the present invention may contain transmembrane domains which span the membrane one or more times. For example, in glycophorin and guanylyl cyclase, the membrane binding region spans the membrane once, whereas the transmembrane domain of rhodopsin spans the membrane seven times, and that of the photosynthetic reaction center of Rhodopseudomonas viridis spans the membrane eleven times (*see* Ross et al., *J. Biol. Chem. 257*:4152, 1982; Garbers, *Pharmac. Ther. 50*:337-345, 1991; Engelman et al., *Proc. Natl. Acad. Sci. USA 77*:2023, 1980; Heijne and Manoil, *Prot. Eng. 4*:109-112, 1990). Membrane anchors for use in the present invention can also include, for example, phosphoinositol anchors. Regardless of the number of times the protein crosses the membrane, the membrane spanning regions typically have a similar structure. More specifically, the 20 to 25 amino-acid residue portion of the domain that is located inside the membrane generally consists almost entirely of hydrophobic residues (*see* Eisenberg et al., *Ann. Rev. Biochem. 53*:595-623, 1984). For example, 28 of the 34 residues in the membrane spanning region of glycophorin are hydrophobic (*see* Ross et al., *supra*; Tomita et al., *Biochemistry 17*:4756-4770, 1978). In addition, although structures such as beta sheets and barrels do occur, the membrane spanning regions typically have an alpha helical structure, as determined by X-ray diffraction, crystallography and cross-linking

studies (*see* Eisenberg et al.,*supra*; Heijne and Manoil, *supra*). The location of these transmembrane helices within a given sequence can often be predicted based on hydrophobicity plots. Stryer et al., *Biochemistry*, 3rd. ed. 304, 1988. Particularly preferred membrane anchors for use within the present invention include naturally occurring cellular proteins (that are non-immunogenic) which have been demonstrated to function as membrane signal anchors (such as glycophorin).

Within a preferred embodiment of the present invention, a DNA sequence is provided which encodes a membrane anchor - gamma interferon fusion protein. Within one embodiment, this fusion protein may be constructed by genetically fusing the sequence which encodes the membrane anchor of the gamma-chain of the Fc receptor, to a sequence which encodes gamma-interferon.

In yet another aspect, recombinant retroviruses are provided which have a therapeutic effect by encoding one or more ribozymes (RNA enzymes) (Haseloff and Gerlach, *Nature 334*:585, 1989) which will cleave, and hence inactivate, RNA molecules corresponding to a pathogenic function. Since ribozymes function by recognizing a specific sequence in the target RNA and this sequence is normally 12 to 17 bp, this allows specific recognition of a particular RNA sequence corresponding to a pathogenic state, such as HIV tat, and toxicity is specific to such pathogenic state. Additional specificity may be achieved in some cases by making this a conditional toxic palliative, as discussed above.

In still another aspect, recombinant retroviruses are provided comprising a biologically active nucleic acid molecule that is an antisense sequence (an antisense sequence may also be encoded by a nucleic acid sequence and then produced within a host cell via transcription). In preferred embodiments, the antisense sequence is selected from the group consisting of sequences which encode influenza virus, HIV, HSV, HPV, CMV, and HBV. The antisense sequence may also be an antisense RNA complementary to RNA sequences necessary for pathogenicity. Alternatively, the biologically active nucleic acid molecule may be a sense RNA (or DNA) complementary to RNA sequences necessary for pathogenicity.

More particularly, the biologically active nucleic acid molecule may be an antisense sequence. Briefly, antisense sequences are designed to bind to RNA transcripts, and thereby prevent cellular synthesis of a particular protein, or prevent use of that RNA sequence by the cell. Representative examples of such sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys. 253*:214-220, 1987; Bzik et al., *PNAS 84*:8360-8364, 1987), antisense HER2 (Coussens et al., *Science 230*:1132-1139, 1985), antisense ABL (Fainstein et al., *Oncogene 4*:1477-1481, 1989), antisense Myc (Stanton et al., *Nature 310*:423-425, 1984) and antisense *ras*, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway.

In addition, within a further embodiment of the invention antisense RNA may be utilized as an anti-tumor agent in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences are believed to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

In another embodiment, the substances of the invention include a surface protein that is itself therapeutically beneficial. For example, in the particular case of HIV, expression of the human CD4 protein specifically in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV env intracellularly could inhibit the formation of viable viral particles much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain membrane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4/HIV env complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-env present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on their cell surfaces).

Still further aspects of the present invention relate to recombinant retroviruses capable of immunostimulation. Briefly, the ability to recognize and defend against foreign pathogens is essential to the function of the immune system. In particular, the immune system must be capable of distinguishing "self" from "nonself" (*i.e.*, foreign), so that the defensive mechanisms of the host are directed toward invading entities instead of against host tissues. Cytolytic T lymphocytes (CTLs) are typically induced, or stimulated, by the display of a cell surface recognition structure, such as a processed, pathogen-specific peptide, in conjunction with a MHC class I or class II cell surface protein.

Diseases suitable to treatment include viral infections such as influenza virus, respiratory syncytial virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hantavirus,

HTLV I, HTLV II and CMV, cancers such as melanomas, renal carcinoma, breast cancer, ovarian cancer and other cancers, and heart disease.

In one embodiment, the invention provides methods for stimulating a specific immune response and inhibiting viral spread by using recombinant retroviruses that direct the expression of an antigen or modified form thereof in susceptible target cells, wherein the antigen is capable of either (1) initiating an immune response to the viral antigen or (2) preventing the viral spread by occupying cellular receptors required for viral interactions. Expression of the protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the recombinant retrovirus is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the correct manner, *i.e.*, in the context of the MHC class I and/or II molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogs thereof (*e.g.*, Altmann et al., *Nature 338*:512, 1989).

An immune response can also be achieved by transferring to an appropriate immune cell (such as a T lymphocyte) (a) the gene for the specific T-cell receptor that recognizes the antigen of interest (in the context of an appropriate MHC molecule if necessary), (b) the gene for an immunoglobulin which recognizes the antigen of interest, or (c) the gene for a hybrid of the two which provides a CTL response in the absence of the MHC context. Thus, recombinant retroviruses may also be used as an immunostimulant, immunomodulator, or vaccine, etc.

In the particular case of disease caused by HIV infection, where immunostimulation is desired, the antigen generated from a recombinant retrovirus may be in a form which will elicit either or both an HLA class I- or class II-restricted immune response. In the case of HIV envelope antigen, for example, the antigen is preferably selected from gp 160, gp 120, and gp 41, which have been modified to reduce their pathogenicity. In particular, the selected antigen is modified to reduce the possibility of syncytia, to avoid expression of epitopes leading to a disease enhancing immune response, to remove immunodominant, but haplotype-specific epitopes or to present several haplotype-specific epitopes, and allow a response capable of eliminating cells infected with most or all strains of HIV. The haplotype-specific epitopes can be further selected to promote the stimulation of an immune response within an animal which is cross-reactive against other strains of HIV. Antigens from other HIV genes or combinations of genes, such as gag, pol, rev, vif, nef, prot, gag/pol, gag prot, etc., may also provide protection in particular cases.

HIV is only one example. This approach should be effective against many virally linked diseases or cancers where a characteristic antigen (which does not need to be a membrane protein) is expressed, such as in HPV and cervical carcinoma, HTLV-I-induced

leukemias, prostate-specific antigen (PSA) and prostate cancer, mutated p53 and colon carcinoma and melanoma, melanoma specific antigens (MAGEs), and melanoma, mucin and breast cancer.

In accordance with the immunostimulation aspects of the invention, substances which are carried and/or expressed by the recombinant retroviruses of the present invention may also include "immunomodulatory factors," many of which are set forth above. Immunomodulatory factors refer to factors that, when manufactured by one or more of the cells involved in an immune response, or, which when added exogenously to the cells, causes the immune response to be different in quality or potency from that which would have occurred in the absence of the factor. The factor may also be expressed from a non-recombinant retrovirus derived gene, but the expression is driven or controlled by the recombinant retrovirus. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, *in vitro* assays which measure cellular proliferation (*e.g.*, $^{3}H$ thymidine uptake), and *in vitro* cytotoxic assays (*e.g.*, which measure $^{51}Cr$ release) (*see*, Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991). Immunomodulatory factors may be active both *in vivo* and *ex vivo*.

Representative examples of such factors include cytokines, such as IL-1, IL-2 (Karupiah et al., *J. Immunology 144*:290-298, 1990; Weber et al., *J. Exp. Med. 166*:1716-1733, 1987; Gansbacher et al., *J. Exp. Med. 172*:1217-1224, 1990; U.S. Patent No. 4,738,927), IL-3, IL-4 (Tepper et al., *Cell 57*:503-512, 1989; Golumbek et al., *Science 254*:713-716, 1991; U.S. Patent No. 5,017,691), IL-5, IL-6 (Brakenhof et al., *J. Immunol. 139*:4116-4121, 1987; WO 90/06370), IL-7 (U.S. Patent No. 4,965,195) , IL-8, IL-9, IL-10, IL-11, IL-12, IL-13 (*Cytokine Bulletin*, Summer 1994), IL-14 and IL-15, particularly IL-2, IL-4, IL-6, IL-12, and IL-13, alpha interferon (Finter et al., *Drugs 42*(5):749-765, 1991; U.S. Patent No. 4,892,743; U.S. Patent No. 4,966,843; WO 85/02862; Nagata et al., *Nature* 284:316-320, 1980; Familletti et al., *Methods in Enz. 78*:387-394, 1981; Twu et al., *Proc. Natl. Acad. Sci. USA 86*:2046-2050, 1989; Faktor et al., *Oncogene 5*:867-872, 1990), beta interferon (Seif et al., *J. Virol. 65*:664-671, 1991), gamma interferons (Radford et al., *The American Society of Hepatology* 20082015, 1991; Watanabe et al., *PNAS 86*:9456-9460, 1989; Gansbacher et al., *Cancer Research 50*:7820-7825, 1990; Maio et al., *Can. Immunol. Immunother. 30*:34-42, 1989; U.S. Patent No. 4,762,791; U.S. Patent No. 4,727,138), G-CSF (U.S. Patent Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), tumor necrosis factors (TNFs) (Jayaraman et al., *J. Immunology 144*:942-951, 1990), CD3 (Krissanen et al., *Immunogenetics 26*:258-266, 1987), ICAM-1 (Altman et al., *Nature 338*:512-514, 1989; Simmons et al., *Nature 331*:624-627, 1988), ICAM-2, LFA-1, LFA-3 (Wallner et al., *J. Exp. Med. 166*(4):923-932, 1987), MHC class I molecules, MHC class II molecules, B7.1-.3, $\beta_2$-microglobulin (Parnes et al., *PNAS 78*:2253-2257, 1981), chaperones such as calnexin,

MHC linked transporter proteins or analogs thereof (Powis et al., *Nature 354*:528-531, 1991). Within one preferred embodiment, the gene encodes gamma-interferon. Immunomodulatory factors may also be agonists, antagonists, or ligands for these molecules. For example soluble forms of receptors can often behave as antagonists for these types of factors, as can mutated forms of the factors themselves.

An example of an immunomodulatory factor cited above is a member of the B7 family of molecules (*e.g.*, B7.1-.3 costimulatory factor). Briefly, activation of the full functional activity of T cells requires two signals. One signal is provided by interaction of the antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen presenting cells. The second signal is required for interleukin-2 (IL-2) production by T cells, and appears to involve interaction of the B7.1-.3 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., *J. Exp. Med.*, *173*:721-730, 1991a and *J. Exp. Med.*, *174*:561-570, 1991). Within one embodiment of the invention, B7.1-.3 may be introduced into tumor cells in order to cause costimulation of $CD8^+$ T cells, such that the $CD8^+$T cells produce enough IL-2 to expand and become fully activated. These $CD8^+$ T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7.1-.3 factor, and, for example, an immunogenic HBV core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will become more effective antigen presenting cells. The HBV core-specific CTL response will be augmented from the fully activated $CD8^+$ T cell via the costimulatory ligand B7.1-.3.

The choice of which immunomodulatory factor to include within a recombinant retrovirus may be based upon known therapeutic effects of the factor, or, experimentally determined. For example, a known therapeutic effector in chronic hepatitis B infections is alpha interferon. This has been found to be efficacious in compensating a patient's immunological deficit, and thereby assisting recovery from the disease. Alternatively, a suitable immunomodulatory factor may be experimentally determined. Briefly, blood samples are first taken from patients with a hepatic disease. Peripheral blood lymphocytes (PBLs) are restimulated *in vitro* with autologous or HLA matched cells (*e.g.*, EBV transformed cells) that have been transduced with a recombinant retrovirus which directs the expression of an immunogenic portion of a hepatitis antigen and the immunomodulatory factor. These stimulated PBLs are then used as effectors in a CTL assay with the HLA matched transduced cells as targets. An increase in CTL response over that seen in the same assay performed using HLA matched stimulator and target cells transduced with a vector encoding the antigen alone, indicates a useful immunomodulatory factor.

Within one embodiment of the invention, the immunomodulatory factor gamma interferon is particularly preferred.

The present invention also includes recombinant retroviruses which encode immunogenic portions of desired antigens including, for example, viral, bacterial or parasite antigens. For example, various immunogenic portions of the HBV S antigens may be combined in order to present an immune response when administered by one of the recombinant retroviruses described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S antigen open reading frame of HBV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S samples are defined as determinant "*a*". Mutually exclusive subtype determinants however have also been identified by two-dimensional double immunodiffusion (Ouchterlony, *Progr. Allergy 5*:1, 1958). These determinants have been designated "*d*" or "*y*" and "*w*" or "*r*" (LeBouvier, *J. Infect. 123*:671, 1971; Bancroft et al., *J. Immunol. 109*:842, 1972; Courouce et al., *Bibl. Haematol. 42*:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two areas of the hepatitis B virus S antigen open reading frame resulting in the following amino acid changes: (1) exchange of lysine-122 to arginine in the hepatitis B virus S antigen open reading frame causes a subtype shift from *d* to *y*, and (2) exchange of arginine-160 to lysine causes the shift from subtype *r* to *w*. In black Africa, subtype *ayw* is predominant, whereas in the U.S. and northern Europe the subtype $adw_2$ is more abundant (*Molecular Biology of the Hepatitis B Virus*, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S antigen open reading frame of HBV virus isolated from individuals within that region.

Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected liver tissue. The polymerase protein consists of at least two domains: the amino terminal domain encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be administered to a warm-blooded animal by introducing into the animal a recombinant retrovirus which expresses the antigen of interest in order to generate an immune response within the animal. Similarly, other HBV antigens such as ORF 5 and ORF 6, (Miller et al., *Hepatology 9*:322-327, 1989), may be expressed utilizing recombinant retroviruses as described herein.

As noted above, at least one immunogenic portion of a hepatitis B antigen can be incorporated into a recombinant retrovirus. The immunogenic portion(s) which are incorporated into the recombinant retrovirus may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long, and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature 351*:290, 1991). From this analysis, peptides may be synthesized and used as targets in an *in vitro* cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays, and proliferation assays.

Within one embodiment of the present invention, at least one immunogenic portion of a hepatitis C antigen can be incorporated into a recombinant retrovirus. Preferred immunogenic portion(s) of hepatitis C may be found in the C and NS3-NS4 regions since these regions are the most conserved among various types of hepatitis C virus (Houghton et al., *Hepatology 14*:381-388, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above for the hepatitis B virus, identification of immunogenic portions of the polypeptide may be predicted based upon amino acid sequence. Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict CTL epitopes. For example, CTL epitopes for the HLA A2.1 haplotype may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature 351:290, 1991)*. From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay.

Within another aspect of the present invention, methods are provided for destroying hepatitis B carcinoma cells comprising the step of administering to a warm-blooded animal a recombinant retrovirus which directs the expression of an immunogenic portion of antigen X, such that an immune response is generated. Sequences which encode the HBxAg may readily be obtained by one of skill in the art given the disclosure provided herein. Briefly, within one embodiment of the present invention, a 642 bp Nco I-Taq I is recovered from ATCC 45020, and inserted into recombinant retroviruses as described above for other hepatitis B antigens.

The X antigen, however, is a known transactivator which may function in a manner similar to other potential oncogenes (*e.g.*, E1A). Thus, it is generally preferable to first alter the X antigen such that the gene product is non-tumorigenic before inserting it into a recombinant retrovirus. Various methods may be utilized to render the X antigen non-tumorigenic including, for example, by truncation, point mutation, addition of premature stop codons, or phosphorylation site alteration. Within one embodiment, the sequence or gene of

interest which encodes the X antigen is truncated. Truncation may produce a variety of fragments, although it is generally preferable to retain greater than or equal to 50% of the encoding gene sequence. In addition, it is necessary that any truncation leave intact some of the immunogenic sequences of the gene product. Alternatively, within another embodiment of the invention, multiple translational termination codons may be introduced into the gene. Insertion of termination codons prematurely terminates protein expression, thus preventing expression of the transforming portion of the protein.

The X gene or modified versions thereof may be tested for tumorigenicity in a variety of ways. Representative assays include tumor formation in nude mice, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

Within another aspect of the present invention, methods are provided for destroying hepatitis C carcinoma cells comprising the step of administering to a warm-blooded animal a recombinant retrovirus which directs the expression of an immunogenic portion of a hepatitis C antigen. Preferred immunogenic portion(s) of a hepatitis C antigen may be found in the polypeptide which contains the Core antigen and the NS1-NS5 regions (Choo et al., *Proc. Natl. Acad. Sci. USA 88*:2451-2455, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above preferred immunogenic portions may be predicted based upon amino acid sequence. Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict CTL epitopes. For example, CTL epitopes for the HLA A2.1 haplotype may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature 351*:290, 1991). Another method that may also be utilized to predict immunogenic portions is to determine which portion has the property of CTL induction in mice utilizing retroviruses (*see*, Warner et al., *AIDS Res. and Human Retroviruses 7*:645-655, 1991). As noted within Warner et al., CTL induction in mice may be utilized to predict cellular immunogenicity in humans. Preferred immunogenic portions may also be deduced by determining which fragments of the polypeptide antigen or peptides are capable of inducing lysis by autologous patient lymphocytes of target cells (*e.g.*, autologous EBV-transformed lymphocytes) expressing the fragments after vector transduction of the corresponding genes.

Preferred immunogenic portions may also be selected in the following manner. Briefly, blood samples from a patient with a target disease, such as HCV, are analyzed with antibodies to individual HCV polypeptide regions (*e.g.*, HCV core, E1, E2/SNI and NS2-NS5 regions), in order to determine which antigenic fragments are present in the patient's serum. In patients treated with alpha interferon to give temporary remission, some antigenic determinants will disappear and be supplanted by endogenous antibodies to the antigen. Such antigens are useful as immunogenic portions within the context of the

present invention (Hayata et al., *Hepatology 13*:1022-1028, 1991; Davis et al., *N. Eng. J. Med. 321*:1501-1506, 1989).

Additional immunogenic portions of a chosen antigen, such as those from the hepatitis B or C virus, may be obtained by truncating the coding sequence. For example, with HBV the following sites may be truncated: Bst UI, Ssp I, Ppu M1, and Msp I (Valenzuela et al., *Nature 280*:815-19, 1979; Valenzuela et al., *Animal Virus Genetics: ICN/UCLA Symp. Mol. Cell Biol.*, 1980, B. N. Fields and R. Jaenisch (eds.), pp. 57-70, New York: Academic). Further methods for determining suitable immunogenic portions as well as methods are also described below in the context of hepatitis C.

With respect to the treatment of HBV, particularly preferred immunogenic portions for incorporation into recombinant retroviruses include HBeAg, HBcAg, and HBsAgs. Further, more than one immunogenic portion (as well as immunomodulatory factors, if desired) may be incorporated into the recombinant retrovirus. For example, within one embodiment a recombinant retrovirus may be prepared which directs the co-expression of both an immunogenic portion of the hepatitis B antigen, as well as an immunogenic portion of the hepatitis C polypeptide. Such constructs may be administered in order to prevent or treat acute and chronic hepatitis infections of either type B or C. Similarly, within other embodiments, a recombinant retrovirus may be prepared which directs the co-expression of both an immunogenic portion of the hepatitis B X antigen, as well as an immunogenic portion of the hepatitis C polypeptide. Such a construct may similarly be administered in order to treat hepatocellular carcinoma that is associated with either hepatitis B or C. In addition, because those individuals chronically infected with hepatitis B and C are at higher risk for developing hepatocellular carcinoma, such a vector may also be utilized as a prophylactic treatment for the disease.

Immunogenic portions may also be selected by other methods. For example, the HLA A2.1/$K^b$ transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T-cell receptor repertoire recognizes the same antigenic determinants recognized by human T-cells. In both systems, the CTL response generated in the HLA A2.1/$K^b$ transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al., *J. Exp. Med. 173*:1007-1015, 1991; Vitiello et al., *Abstract of Molecular Biology of Hepatitis B Virus Symposia*, 1992).

Immunogenic proteins of the present invention may also be manipulated by a variety of methods known in the art, in order to render them more immunogenic. Representative examples of such methods include: adding amino acid sequences that correspond to T helper epitopes; promoting cellular uptake by adding hydrophobic residues; by forming particulate structures; or any combination of these (*see generally*, Hart, op. cit.,

Milich et al., *Proc. Natl. Acad. Sci. USA 85*:1610-1614, 1988; Willis, *Nature 340*:323-324, 1989; Griffiths et al., *J. Virol. 65*:450-456, 1991).

The present invention also includes compositions and methods for treating, as well as vaccines for preventing, various feline diseases, including for example feline leukemia virus ("FeLV") and feline immunodeficiency virus ("FIV") infections. These viruses are discussed more fully in PCT application number US 93/09070.

Briefly, feline leukemia virus (FeLV) is a retrovirus of the oncornavirus subfamily. FeLV is presently believed to exist in three subgroups - A, B or C - which are differentiated by their envelope antigens gp70 and p15E. FeLV is also comprised of a number of core antigens, including p15, p12, p27, and p10, which are highly conserved for all subgroups of FeLV (*see* Geering et al., *Vir. 36*:678-680, 1968; Hardy et al., *JAVMA 158*:1060-1069, 1971; Hardy et al., *Science 166*:1019-1021, 1969). Within one embodiment of the invention, the recombinant retrovirus directs the expression of at least one portion of a feline leukemia virus antigen selected from the group consisting of p15*gag*, p12*gag*, p27*gag*, p10*gag*, p14*pol*, p80*pol*, p46*pol*, gp70*env*, and p15*env*. Within a particularly preferred embodiment, the recombinant retrovirus directs the expression of gp85*env*. Sequences which encode these antigens may be readily obtained given the disclosure provided herein (*see* Donahue et al., *J. Vir. 62*(3):722-731, 1988; Stewart et al., *J. Vir. 58*(3):825-834, 1986; Kumar et al., *J. Vir. 63*(5):2379-2384, 1989; Elder et al., *J. Vir. 46*(3):871-880, 1983; Berry et al., *J. Vir. 62*(10):3631-3641, 1988; Laprevotte et al., *J. Vir. 50*(3):884-894, 1984).

Feline immunodeficiency virus (FIV) has been classified as a retrovirus of the lentivirus subfamily, based upon the magnesium requirement for reverse transcriptase (RT) and the morphology of viral particles (*see* Pederesen et al., *Science 235*:790-793, 1987). The feline immunodeficiency virus is morphologically and antigenically distinct from other feline retroviruses, including feline leukemia virus, type C oncorna virus (RD-114), and feline syncytium-forming virus (FeSFV) (*see* Yamamoto et al., "Efficacy of experimental FIV vaccines, (Abstract), First International Conference of Feline Immunodeficiency Virus Researchers, University of California, Davis, CA, Sep. 4-7, 1991). Within one embodiment of the invention, the recombinant retrovirus directs the expression of at least one immunogenic portion of an feline immunodeficiency virus antigen selected from the group consisting of p15*gag*, p24*gag*, p10*gag*, p13*pol*, p62*pol*, p15*pol* and p36*pol*. Within a particularly preferred embodiment, the recombinant retrovirus directs the expression of gp68*env*, gp27*env* and *rev*. Within the context of the present invention, "*rev*" is understood to refer to the antigen corresponding to the *rev* open reading frame (*see*, Phillips et al., First International Conference, *supra*). Sequences which encode these antigens may be readily obtained by one of skill in the art given the disclosure provided herein (*see* Phillips et al., *J.*

*Vir.* *64*(10):4605-4613, 1990; Olmsted et al., *PNAS* *86*:2448-2452, 1989; Talbott et al., *PNAS* *86*:5743-5747, 1989).

Still other examples include recombinant retroviruses which direct the expression of a non-tumorigenic, altered genes such as the ras (ras*) gene (*see* WO 93/10814). Briefly, the ras* gene is an attractive target because it is causally linked to the neoplastic phenotype, and indeed may be necessary for the induction and maintenance of tumorigenesis in a wide variety of distinct cancers, such as pancreatic carcinoma, colon carcinoma and lung adenocarcinoma. In addition, ras* genes are found in pre-neoplastic tumors, and therefore immune intervention therapy may be applied prior to detection of a malignant tumor.

Normal ras genes are non-tumorigenic and ubiquitous in all mammals. They are highly conserved in evolution and appear to play an important role in maintenance of the cell cycle and normal growth properties. The normal ras protein is a G-protein which binds GTP and has GTPase activity, and is involved in transmitting signals from the external milieu to the inside of the cell, thereby allowing a cell to respond to its environment. Ras* genes on the other hand alter the normal growth regulation of neoplastic cells by uncoupling cellular behavior from the environment, thus leading to the uncontrolled proliferation of neoplastic cells. Mutation of the ras gene is believed to be an early event in carcinogenesis (Kumar et al., "Activation of ras Oncogenes Preceding the Onset of Neoplasia," *Science* *248*:1101-1104, 1990), which, if treated early, may prevent tumorigenesis.

Ras* genes occur in a wide variety of cancers, including for example, pancreatic, colon, and lung adenocarcinomas. However, the spectrum of mutations occurring in the ras* genes found in a variety of cancers is quite limited. These mutations alter the GTPase activity of the ras protein by converting the normal on/off switch to a constitutive ON position. Tumorigenic mutations in ras* occur primarily (*in vivo*) in only 3 codons: 12, 13 and 61. Codon 12 mutations are the most prevalent in both human and animal tumors.

Within another embodiment of the present invention, recombinant retroviruses are provided which direct the expression of an altered p53 (p53*) gene. Briefly, p53 is a nuclear phosphoprotein which was originally discovered in extracts of transformed cells, and thus was initially classified as an oncogene (Linzer and Levine, *Cell* *17*:43-52, 1979; Lane and Crawford, *Nature* *278*:261-263, 1979). It was later discovered that the original p53 cDNA clones were mutant forms of p53 (Hinds et al., *J. Virol.* *63*:739-746, 1989). It now appears that p53 is a tumor suppressor gene, which negatively regulates the cell cycle, and that mutation of this gene may lead to tumor formation. Of colon carcinomas that have been studied, 75%-80% show a loss of both p53 alleles, one through deletion, and

the other through point mutation. Similar mutations are found in lung cancer, and in brain and breast tumors.

The majority of p53 mutations (*e.g.*, $p53^{*1}$, $p53^{*2}$, etc.) are clustered between amino-acid residues 130 to 290 (*see* Levine et al., *Nature 351*:453-456, 1991; *see also* the following references which describe specific mutations in more detail: Baker et al., *Science 244*:217-221, 1989; Nigro et al., *Nature 342*:705-708, 1989 (p53 mutations cluster at four "hot spots" which coincide with the four highly conserved regions of the genes and these mutations are observed in human brain, breast, lung and colon tumors); Vogelstein, *Nature 348*:681-682, 1990; Takahashi et al., *Science 246*:491-494, 1989; Iggo et al., *Lancet 335*:675-679, 1990; James et al., *Proc. Natl. Acad. Sci. USA 86*:2858-2862, 1989; Mackay et al., *Lancet 11*:1384-1385,1988; Kelman et al., *Blood 74*:2318-2324, 1989; Malkin et al., *Science 250*:1233-1238, 1990; Baker et al., *Cancer Res. 50*:7717-7722, 1991; Chiba et al., *Oncogene 5*:1603-1610, 1990 (pathogenesis of early stage non-small cell lung cancer is associated with somatic mutations in the p53 gene between codons 132 to 283); Prosser et al., *Oncogene 5*:1573-1579, 1990 (mutations in the p53 gene coding for amino acids 126 through 224 were identified in primary breast cancer); Cheng and Hass, *Mol. Cell. Biol. 10*:5502-5509, 1990; Bartek et al., *Oncogene 5*:893-899, 1990; Rodrigues et al., *Proc. Natl. Acad. Sci. USA 87*:7555-7559, 1990; Menon et al., *Proc. Natl. Acad. Sci. USA 87*:5435-5439, 1990; Mulligan et al., *Proc. Natl. Acad. Sci. USA 87*:5863-5867, 1990; and Romano et al., *Oncogene 4*:1483-1488, 1990 (identification of a p53 mutation at codon 156 in human osteosarcoma derived cell line HOS-SL)).

Certain alterations of the p53 gene may be due to certain specific toxins. For example, Bressac et al. (*Nature 350*:429-431, 1991) describes specific G to T mutations in codon 249, in patients affected with hepatocellular carcinoma. One suggested causative agent of this mutation is aflatoxin $B_1$, a liver carcinogen which is known to be a food contaminant in Africa.

Four regions of the gene that are particularly affected occur at residues 132-145, 171-179, 239-248, and 272-286. Three "hot spots" of particular interest occur at residues 175, 248 and 273 (Levine et al., *Nature 351*:453-456, 1991). These alterations as well as others which are described above result in the production of protein(s) which contain novel coding sequence(s). The novel proteins encoded by these sequences may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequence ($p53^*$).

Within another embodiment of the present invention, recombinant retroviruses are provided which direct the expression of an altered Rb ($Rb^*$) gene. Briefly, retinoblastoma is a childhood eye cancer associated with the loss of a gene locus designated Rb, which is located in chromosome band 13q14. A gene from this region has been cloned

which produces a nuclear phosphoprotein of about 110 kd (Friend et al., *Nature 323*:643, 1986; Lee et al., *Science 235*:1394, 1987; and Fung et al., *Science 236*:1657, 1987).

Rb is believed to be a negative regulator of cellular proliferation, and has a role in transcriptional control and cell-cycle regulation. Rb binds to at least seven proteins found in the nucleus, and in particular, appears to be involved with a cellular transcription factor which has been designated both E2F (Bagchi et al., *Cell 62*:659-669, 1990) and DRTF (Shivji and La Thangue, *Mol. Cell. Biol. 11*:1686-1695, 1991). Rb is believed to restrict cellular growth by sequestering a variety of nuclear proteins involved in cellular proliferation.

Deletions within the Rb gene have been detected which evidence that the Rb gene may be responsible for tumorigenicity. These deletions include, for example, a deletion in exon 21 in a prostate cancer and bladder cancer cell line (Bookstein et al., *Science 247*:712-715, 1990; Horowitz et al., *Science 243*:937, 1989), a deletion of exon 16 in a small-cell carcinoma of the lung (Shew et al., *Cell Growth and Diff. 1*:17, 1990), and a deletion between exons 21 and 27 (Shew et al., *Proc. Natl. Acad. Sci. USA 87*:6, 1990). Deletion of these exons results in the production of a protein containing a novel coding sequence at the junction of the deleted exons. This novel protein coding sequence may be used as a marker of tumorigenic cells, and an immune response directed against this novel coding region may eliminate tumorigenic cells containing the Rb exon deletion.

Within another embodiment of the present invention, recombinant retroviruses are provided which direct the expression of an altered gene which causes Wilms' tumor. Briefly, Wilms' tumor is typically found in children younger than 16 years of age. One child in 10,000 will develop this tumor, which comprises approximately 5% of childhood cancers. The tumor usually presents itself as a large abdominal mass which is surrounded by a fibrous pseudocapsule. Approximately 7% of the tumors are multifocal in one kidney, and 5.4% are involved with both kidneys. The Wilms' tumor gene has been localized to chromosome 11p13, and a cDNA clone (wt1) has been isolated that is characteristic of a tumor suppressor gene (Call et al., *Cell 60*:509, 1990; Gessler et al., *Nature 343*:744, 1990; Rose et al., *Cell 60*:495, 1990; and Haber et al., *Cell 61*:1257, 1990). The wt1 gene encodes a protein which contains four zinc fingers and a glutamine and proline rich amino terminus. Such structures are believed to be associated with transcriptional and regulatory functions.

Mutations of the Wilms' tumor gene include the insertion of lysine, threonine, and serine between the third and forth zinc fingers. A wt1 protein which contains such insertions does not bind to the EGR-1 site. A second alternative mutation results in the insertion of about 17 amino acids in the region immediately $NH_2$-terminal to the zinc finger domain (Madden et al., *Science 253*:1550-1553, 1991; Call et al., *Cell 60*:509, 1990; Gessler

et al., *Nature 343*:744, 1990; Rose et al., *Cell 60*:495, 1990; Haber et al., *Cell 61*:1257, 1990; and Buckler et al., *Mol. Cell. Biol. 11*:1707, 1991).

Within another embodiment of the present invention, recombinant retroviruses are provided which direct the expression of an altered mucin. Briefly, mucins are large molecular weight glycoproteins which contain approximately 50% carbohydrate. Polymorphic epithelial mucin (PEM) is a tumor-associated mucin (Girling et al., *Int. J. Cancer 43*:1072-1076, 1989) which is found in the serum of cancer patients. The full-length cDNA sequence has been identified (Gendler et al., *J. Biol. Chem. 265*(25):15286-15293, 1990; Lan et al., *J. Biol. Chem. 265*(25):15294-15299, 1990; and Ligtenberg et al., *J. Biol. Chem. 265*:5573-5578, 1990). Breast tumors and pancreatic tumors both express a mucin with an identical core sequence, containing a 20 amino-acid tandem repeat (Jerome et al., *Cancer Res. 51*:2908-2916, 1991). CTL lines which have been developed to breast tumors which cross-react with pancreatic tumor targets, and further appear to specifically recognize the specific 20 amino-acid tandem repeat (Jerome et al., *supra*). A sequence encoding one or more of the 20 amino-acid tandem repeats may be expressed by a recombinant retrovirus of the present invention, in order to develop an immune response against tumor cells which contain this sequence.

Within another embodiment of the present invention, recombinant retroviruses are provided which direct the expression of an altered DCC (deleted in colorectal carcinomas) gene. Briefly, a very common region of allelic loss in colorectal tumors is chromosome 18q, which is lost in more than 70% of carcinomas, and in almost 50% of late adenomas. A presumptive tumor suppressor gene (DCC) from this region has been identified (Fearon et al., 1990), which encodes a protein with significant homology to cell-surface adhesion molecules, such as neural cell-adhesion molecule (NCAM) and contactin (reviewed by Edelman in *Biochem 27*:3533-3543, 1988). This protein is believed to play a role in the development of colorectal tumors, perhaps through alterations in normal cell-cell and/or cell-extracellular matrix interactions.

The DCC gene is expressed in normal colonic mucosa, but its expression is reduced or absent in the majority of colorectal carcinomas (Solomon, *Nature 343*:412-414, 1990). This loss of expression has been associated in some cases with somatic mutations of the DCC gene. A contiguous stretch of DNA comprising 370 kb has been cloned which encodes an approximately 750 amino acid protein (Fearon et al., "Identification of a Chromosome 18q Gene That Is Altered in Colorectal Cancers," *Science 247*:49-56, 1990).

Within another embodiment of the present invention, recombinant retroviruses are provided which direct the expression of MCC (mutated in colorectal cancer) or APC. Both MCC and APC have been identified as tumor suppressor genes (Kinzler et al., *Science 251*:1366-1370, 1991) which undergo mutation in familial adenomatous polyposis

(FAP). FAP is believed to be the most common autosomal dominant disease which leads to cancer, and it affects at least 1 in 5,000 individuals in the United States (Nishiho et al., *Science 253*:665-669, 1991). Affected individuals usually develop hundreds to thousands of adenomatous polyps of the colon and rectum, which may progress to carcinoma. Gardner's syndrome ("GS," a variant of FAP) presents desmoid tumors, osteomas, and other neoplasms together with multiple adenomas of the colon and rectum. This proliferation is believed to be induced by loss or inactivation of the familial adenomatous polyposis gene (and in particular, MCC and APC) which is found on chromosome 5q.

For example, in Nishiho et al. (*supra*), the following germ line mutations of the APC gene were found in FAP and GS patients: (1) Codon 280, a serine to stop mutation (in a patient with mandibular osteoma), (2) codon 302, an arginine to stop mutation in two separate patients, one with a desmoid tumor, (3) codon 414, an arginine to cysteine mutation in a patient with mandibular osteoma, and (5) codon 713, a serine to stop mutation in another patient with mandibular osteoma (Nishiho et al., *Science 253*:665-669, 1991). In addition, six point mutations were identified in MCC codon numbers 12, 145, 267, 490, 506, and 698, as well as an additional 4 somatic mutations in APC (codons number 289, 332, 438, and 1338).

Within other embodiments of the invention, recombinant retroviruses are provided which direct the expression of an altered receptor which is functionally locked or stuck in an "ON" or "OFF" mode. Briefly, many cellular receptors are involved in cell growth by monitoring the external environment and signaling the cell to respond appropriately. If either the monitoring or signaling mechanisms fail, the cell will no longer respond to the external environment and may exhibit uncontrolled growth. Many different receptors or receptor-like structures may function as altered cellular components, including, for example, neu and mutated or altered forms of the thyroid hormone receptor, the PDGF receptor, the insulin receptor, the Interleukin receptors (*e.g.*, IL-1, -2, -3, etc. receptors), or the CSF receptors, such as the G-CSF, GM-CSF, or M-CSF receptors.

For example, neu (also referred to as the Human Epidermal Growth Factor Receptor "HER" or the Epidermal Growth Factor "EGF" receptor) is an altered receptor which is found in at least 28% of women with breast cancer. A cDNA clone which encodes this protein has been isolated (Slamon et al., *Science 244*:707-712, 1989; Slamon et al., *Cancer Cells 7*:371-380, 1989; Shih et al., *Nature 290*:261, 1981). This clone encodes a protein that has extracellular, transmembrane, and intracellular domains (Schechter, *Nature 312*:513, 1984; Coussens et al., *Science 230*:1132, 1985) and thus is believed to encode the neu receptor.

Studies of the rat neu gene isolated from chemically induced neuroglioblastoma cells indicate that it contains a single mutation at position 664 from valine

to glutamic acid (Bargmann et al., *EMBO J. 7*:2043, 1988). In other studies, baby rats which were treated with N-ethyl-N-nitrosourea developed malignant tumors of the nervous system. All 47 trigeminal schwannomas and 12 neurinomas which developed carried a T to A transversion at position 664 of the neu gene (Nikitin et al., *Proc. Natl. Acad. Sci USA 88*:9939-9943, 1991).

Other altered receptors may also be expressed by recombinant retroviruses in order to destroy selected tumor cells. For example, a deletion in chromosome 3p21-p25 has been associated with small-cell lung carcinomas (Leduc et al., *Am. J. Hum. Genet. 44*:282-287, 1989). A deletion is believed to occur in the ERBAb gene which otherwise codes for a DNA-binding thyroid hormone receptor (THR).

Alterations in receptors as described above result in the production of protein(s) (or receptors) containing novel coding sequence(s). The novel protein(s) encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding region(s) may be utilized to destroy tumorigenic cells containing the altered sequence(s) or gene(s).

If the altered cellular component is associated with making the cell tumorigenic, then, it is necessary to make the altered cellular component non-tumorigenic. For example, within one embodiment, the sequence or gene of interest which encodes the altered cellular component is truncated in order to render the gene product non-tumorigenic. The gene encoding the altered cellular component may be truncated to a variety of sizes, although it is preferable to retain as much as possible of the altered cellular component. In addition, it is necessary that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational termination codons may be introduced into the gene which encodes the altered cellular component, downstream of the immunogenic region. Insertion of termination codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

Within one embodiment, the ras* gene is truncated in order to render the ras* protein non-tumorigenic. Briefly, the carboxy-terminal amino acids of ras* functionally allow the protein to attach to the cell membrane. Truncation of these sequences renders the altered cellular component non-tumorigenic. Preferably, the ras* gene is truncated in the purine ring formation, for example around the sequence which encodes amino acid number 110. The ras* gene sequence may be truncated such that as little as about 20 amino acids (including the altered amino acid(s) are encoded by the recombinant retrovirus, although preferably, as many amino acids as possible should be expressed (while maintaining non-tumorigenicity).

Within another embodiment, the p53* protein is modified by truncation in order to render the cellular component non-tumorigenic. As noted above, not all mutations

of the p53 protein are tumorigenic, and therefore, not all mutations would have to be truncated. Nevertheless, within a preferred embodiment, p53* is truncated to a sequence which encodes amino acids 100 to 300, thereby including all four major "hot spots."

Other altered cellular components which are oncogenic may also be truncated in order to render them non-tumorigenic. For example, both neu and bcr/abl may be truncated in order to render them non-tumorigenic. Non-tumorigenicity may be confirmed by assaying the truncated altered cellular component as described above.

It should be noted, however, that if the altered cellular component is only associated with non-tumorigenic cells in general, and is not required or essential for making the cell tumorigenic, then it is not necessary to render the cellular component non-tumorigenic. Representative examples of such altered cellular components which are not tumorigenic include Rb*, ubiquitin*, and mucin*.

As noted above, in order to generate an appropriate immune response, the altered cellular component must also be immunogenic. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes often possess an immunogenic amphipathic alpha-helix component. In general, however, it is preferable to determine immunogenicity in an assay. Representative assays include an ELISA which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells such as gamma-interferon assays, IL-2 production assays, and proliferation assays. A particularly preferred method for determining immunogenicity is the CTL assay.

Once a sequence encoding at least one anti-tumor agent has been obtained, it is preferable to ensure that the sequence encodes a non-tumorigenic protein. Various assays are known and may easily be accomplished which assess the tumorigenicity of a particular cellular component. Representative assays include tumor formation in nude mice or rats, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

For this and many other aspects of the invention, tumor formation in nude mice or rats is a particularly important and sensitive method for determining the tumorigenicity of an anti-tumor agent. Nude mice lack a functional cellular immune system (*i.e.*, do not possess CTLs), and therefore provide a useful *in vivo* model in which to test the tumorigenic potential of cells. Normal non-tumorigenic cells do not display uncontrolled growth properties if injected into nude mice. However, transformed cells will rapidly proliferate and generate tumors in nude mice. Briefly, in one embodiment the recombinant retrovirus is delivered to syngeneic murine cells, followed by administration into nude mice. The mice are visually examined for a period of 2 to 8 weeks after administration in order to determine tumor growth. The mice may also be sacrificed and autopsied in order to determine whether tumors are present. (Giovanella et al., *J. Natl. Cancer Inst.* *48*:1531-1533, 1972; Furesz et al., "Tumorigenicity testing of cell lines considered for production of

biological drugs," *Abnormal Cells*, New Products and Risk, Hopps and Petricciani (eds.), Tissue Culture Association, 1985; and Levenbook et al., *J. Biol. Std. 13*:135-141, 1985). Tumorigenicity may also be assessed by visualizing colony formation in soft agar (MacPherson and Montagnier, *Vir. 23*:291-294, 1964). Briefly, one property of normal non-tumorigenic cells is "contact inhibition" (*i.e.*, cells will stop proliferating when they touch neighboring cells). If cells are plated in a semi-solid agar support medium, normal cells rapidly become contact inhibited and stop proliferating, whereas tumorigenic cells will continue to proliferate and form colonies in soft agar.

Transgenic animals, such as transgenic mice, may also be utilized to assess the tumorigenicity of an anti-tumor agent (*e.g.*, Stewart et al., *Cell 38*:627-637, 1984; Quaife et al., *Cell 48*:1023-1034, 1987; and Koike et al., *Proc. Natl. Acad. Sci. USA 86*:5615-5619, 1989). In transgenic animals, the gene of interest may be expressed in all tissues of the animal. This unregulated expression of the transgene may serve as a model for the tumorigenic potential of the newly introduced gene.

In addition to tumorigenicity studies, it is generally preferable to determine the toxicity of the recombinant retroviruses, prior to administration. A variety of methods well known to those of skill in the art may be utilized to measure such toxicity, including for example, clinical chemistry assays which measure the systemic levels of various proteins and enzymes, as well as blood cell volume and number.

The present invention also provides recombinant retroviruses capable of immune down-regulation. Briefly, specific down-regulation of inappropriate or unwanted immune responses, such as in autoimmune or pseudo-autoimmune diseases such as chronic hepatitis, diabetes, rheumatoid arthritis, graft vs. host disease and Alzheimer's, or in transplants of heterologous tissue such as bone marrow, can be engineered using immune-suppressive viral gene products, or active portion thereof, which suppress surface expression of transplantation (MHC) antigen. Within the present invention, an "active portion" of a gene product is that fragment of the gene product which must be retained for biological activity. Such fragments or active domains can be readily identified by systematically removing nucleotide sequences from the protein sequence, transforming target cells with the resulting recombinant retrovirus, and determining MHC class I presentation on the surface of cells using FACS analysis or other immunological assays, such as a CTL assay. These fragments are particularly useful when the size of the sequence encoding the entire protein exceeds the capacity of the viral carrier. Alternatively, the active domain of the MHC antigen presentation inhibitor protein can be enzymatically digested and the active portion purified by biochemical methods. For example, a monoclonal antibody that blocks the active portion of the protein can be used to isolate and purify the active portion of the cleaved protein (Harlow et al., *Antibodies: A Laboratory Manual*, Cold Springs Harbor, 1988).

Within one embodiment, the suppression is effected by specifically inhibiting the activation of display of processed peptides in the context of self MHC molecules along with accessory molecules such as CD8, intercellular adhesion molecule -1 (ICAM-1), ICAM-2, ICAM-3, leukocyte functional antigen-1 (LFA-1) (Altmann et al., *Nature 338*:521, 1989), the B7.1-.3 molecule (Freeman et al., *J. Immunol. 143*:2714, 1989), LFA-3 (Singer, *Science 255*:1671, 1992; Rao, *Crit. Rev. Immunol. 10*:495, 1991), or other cell adhesion molecules. Antigenic peptide presentation in association with MHC class I molecules leads to CTL activation. Transfer and stable integration of specific sequences capable of expressing products expected to inhibit MHC antigen presentation block activation of T-cells, such as $CD8^+$ CTL, and therefore suppress graft rejection. A standard CTL assay may be utilized in order to detect this response. Components of the antigen presentation pathway include the 45 Kd MHC class I heavy chain, $\beta_2$-microglobulin, processing enzymes such as proteases, accessory molecules, chaperones such as calnexin (Gaczynska, *et al.*, *Nature*, *365*: 264-282, 1993), and transporter proteins such as PSF1, TAP1 and TAP 2 (Driscoll, *et al.*, *Nature*, *365*: 262-263, 1993).

In an alternative example, recombinant retroviruses are provided which direct the expression of a gene product or an active portion of a gene product capable of binding $\beta_2$-microglobulin. Briefly, transport of MHC class I molecules to the cell surface for antigen presentation requires association with $\beta_2$-microglobulin. Thus, proteins that bind $\beta_2$-microglobulin and inhibit its association with MHC class I indirectly inhibit MHC class I antigen presentation. Suitable proteins include the H301 gene product. Briefly, the H301 gene, obtained from the human cytomegalovirus (CMV) encodes a glycoprotein with sequence homology to the $\beta_2$-microglobulin binding site on the heavy chain of the MHC class I molecule (Browne et al., *Nature 347*:770, 1990). H301 binds $\beta_2$-microglobulin, thereby preventing the maturation of MHC class I molecules, and renders transformed cells unrecognizable by cytotoxic T-cells, thus evading MHC class I restricted immune surveillance.

Within another embodiment, recombinant retroviruses are provided which direct the expression of a protein or active portion of a protein that binds to newly synthesized MHC class I molecules intracellularly. This binding prevents migration of the MHC class I molecule from the endoplasmic reticulum, resulting in the inhibition of terminal glycosylation. This blocks transport of these molecules to the cell surface and prevents cell recognition and lysis by CTL. For instance, one of the products of the E3 gene may be used to inhibit transport of MHC class I molecules to the surface of the transformed cell. More specifically, E3 encodes a 19 kD transmembrane glycoprotein, E3/19K, transcribed from the E3 region of the adenovirus 2 genome. Within the context of the present invention, tissue cells are transformed with a recombinant retrovirus containing the E3/19K sequence, which

upon expression produces the E3/19K protein. The E3/19K protein inhibits the surface expression of MHC class I surface molecules, and cells transformed by the recombinant retrovirus evade an immune response. Consequently, donor cells can be transplanted with reduced risk of graft rejection and may require only a minimal immunosuppressive regimen for the transplant patient. This allows an acceptable donor-recipient chimeric state to exist with fewer complications. Similar treatments may be used to treat the range of so-called autoimmune diseases, including systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis or chronic hepatitis B infection.

Another alternative method of immunosuppression involves the use of antisense message, ribozyme, or other gene expression inhibitor specific for T-cell clones which are autoreactive in nature. These block the expression of the T-cell receptor of particular unwanted clones responsible for an autoimmune response. The anti-sense, ribozyme, or other gene may be introduced using a viral vector delivery system.

Other proteins, not discussed above, that function to inhibit, suppress or down-regulate MHC class I antigen presentation may also be identified and utilized within the context of the present invention. In order to identify such proteins, in particular those derived from mammalian pathogens (and, in turn, active portions thereof), a recombinant retrovirus that expresses a protein or an active portion thereof suspected of being capable of inhibiting MHC class I antigen presentation is transformed into a tester cell line, such as BC. The tester cell lines with and without the sequence encoding the candidate protein are compared to stimulators and/or targets in the CTL assay. A decrease in cell lysis corresponding to the transformed tester cell indicates that the candidate protein is capable of inhibiting MHC presentation.

Many infectious diseases, cancers, autoimmune diseases, and other diseases involve the interaction of viral particles with cells, cells with cells, or cells with factors. In viral infections, viruses commonly enter cells via receptors on the surface of susceptible cells. In cancers, cells may respond inappropriately or not at all to signals from other cells or factors. In autoimmune disease, there is inappropriate recognition of "self" markers. Within the present invention, such interactions may be blocked by utilizing recombinant retroviruses that produce, *in vivo*, an analogue to either of the partners in an interaction. Such an analogue is known as a blocking agent.

This blocking action may occur intracellularly, on the cell membrane, or extracellularly. The blocking action of a viral or, in particular, a recombinant retrovirus carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

For example, in the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker

would be a recombinant retrovirus expressing either an HIV env analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Delivery of a recombinant retrovirus encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule.

Vector particles leading to expression of HIV env may also be constructed. It will be evident to one skilled in the art which portions are capable of blocking virus adsorption without overt pathogenic side effects (Willey et al., *J. Virol. 62*:139, 1988; Fisher et al., *Science 233*:655, 1986).

Another aspect of the invention involves the delivery of suppressor genes which, when deleted, mutated or not expressed in a cell type, lead to tumorigenesis in that cell type. Reintroduction of the deleted gene by means of a viral vector leads to regression of the tumor phenotype in these cells. Since malignancy can be considered to be an inhibition of cellular terminal differentiation compared with cell growth, the delivery and expression of gene products which lead to differentiation of a tumor should also, in general, lead to regression.

Sequences which encode the above-described nucleic acid molecules may be obtained from a variety of sources. For example, plasmids which contain sequences that encode altered cellular products may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as Advanced Biotechnologies (Columbia, Maryland). Representative examples of plasmids containing some of the above-described sequences include ATCC No. 41000 (containing a G to T mutation in the 12th codon of ras), and ATCC No. 41049 (containing a G to A mutation in the 12th codon).

Other nucleic acid molecules that encode the above-described substances, as well as other nucleic acid molecules that are advantageous for use within the present invention, may be readily obtained from a variety of sources, including for example depositories such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Representative examples include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No 67024 (which

contains a sequence which encodes Interleukin-1b), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

Molecularly cloned genomes which encode the hepatitis B virus may be obtained from a variety of sources including, for example, the American Type Culture Collection (ATCC, Rockville, Maryland). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (*see* Figure 3 of Blum et al., *TIG 5*(5):154-158, 1989) in the Bam HI site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA 78*:2606-2610, 1981). (Note that correctable errors occur in the sequence of ATCC No. 45020.)

Alternatively, cDNA sequences for use with the present invention may be obtained from cells which express or contain the sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (*see* U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159. See also *PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press, 1989) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Nucleic acid molecules which are carried and/or expressed by the recombinant retroviruses described herein may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, APB DNA synthesizer model 392 (Foster City, California).

C. <u>Pretreatment of target tissues</u>:

Retroviral vectors are known to preferentially infect dividing cells. (See Miller, et al., (1990) Molec. Cell Biol. 10:4239.) There are variety of techniques that may be used to increase the number of dividing cells in target tissues and thereby enhance the efficiency of target cell infection by the retroviral vectors of the invention. For example,

growth factors may be used to stimulate target tissues to enter the portion of the cell cycle in which retrovector integration can take place. Such growth factors and their target tissues include, but are not limited to, the following:

Protein S and Gas6 acting on nervous system cells and smooth muscle cells; thrombin acting on smooth muscle cells, gastrointestinal epithelium, liver fat storage cells, dental pulp cells, fibroblasts, endothelial cells, mesangial cells, and astrocytes; coagulation Factor Xa acting on smooth muscle cells; nerve growth factor acting on nervous system cells; CSF-1 acting on placenta and endometrium; IGF-1 acting on kidney, bone, skin, adipose tissue, airway smooth muscle cells, gastrointestinal epithelium, neural tissue, muscle, and follicular cells; insulin acting on gastrointestinal epithelium, skin, and adipose tissue; KGF acting on urothelium, mammary epithelium, skin, liver, and gastrointestinal epithelium; TGF acting on gastrointestinal epithelium, dental pulp, neural tissue, fibroblasts, connective tissue, inner ear sensory epithelium, colon, bone, pneumocyte type II cells, cornea, and smooth muscle cells; endothelin acting on kidney, smooth muscle cells, melanocytes, cardiac muscle, and astrocytes; PDGF acting on kidney, airway smooth muscle cells, gastrointestinal epithelium, neural tissue, and connective tissue; EGF acting on kidney, skin, neural tissue, inner ear sensory epithelium, connective tissue, fibroblasts, endometrium, liver, and intestine; HGF acting on liver, kidney, mammary epithelium, gastrointestinal epithelium, alveolar epithelium, melanocytes, placenta, and alveolar type II cells; PSA acting on prostate, breast, lung, colon, ovary, liver, and kidney; injurin and HGF-activators acting on liver, kidney, and mammary epithelium; FGF acting on neural cells, kidney, endothelium, fibroblasts, skin, skeletal muscle, connective tissue, melanocytes, cornea, bone marrow, dental pulp cells, liver, melanocytes, smooth muscle cells, and thyroid follicular cells; VEGF acting on endothelial cells; Arg-vasopressin acting on liver, kidney, and fibroblasts; thyroid hormones acting on bone; azoxymethane acting on the colon; prostaglandins acting on liver and dental pulp; IL1 acting on fibroblasts; IL2 acting on T cells; IL15 acting on muscle; triiodothyronine acting on liver; LIF acting on muscle and bone; amphiregulin acting on the skin; soluble thrombomodulin acting on fibroblasts; stem cell factor acting on erythroid progenitors; osteogenic protein 1 acting on chondrocytes, and bone; bone morphogenic protein acting on liver; MGF acting on melanocytes; MGSA acting on melanocytes; heregulins acting on mammary epithelium, keratinocytes, and Schwann cells; and melanotropin acting on melanocytes. Growth factors can also be used in combination, particularly but not limited to mixtures consisting of one or several of EGF, IGF, PDGF, FGF, or KGF. In particular, multiple growth factors known to stimulate cell division of a particular target tissue can used in comination to increase the proportion of dividing cells in the tissue. The actions of the above and other growth factors can also be potentiated with substances including but not limited to dextan sulfate, heparin, and other sulfated glycosaminoglycans, FBP, leukotrienes, prostaglandins, oleic acid, HGF activators, androgens, estrogens, ethanol, PF4, and TGF beta antagonists. Treatment with growth

factors or the other substances described above can occur by adminsitering the substances in vivo or can also be used in other treatment modalities such as ex vivo treatment.

Liver is an attractive organ for gene therapy because it is easily accessible via the circulation and is the source of a varitey of proteins involved in genetic disorders, including factor VIII. Gene therapy targeting the liver using the retroviral vectors of the invention can be performed with or without pretreatment to incude benign hyperplasia of the liver. Pretreatment to induce benign liver hyperplasia can be effected, for example, by treatment with hepatic growth factor (hGF) and/or transforming growth factor alpha. (See eg. Liu, et al. (1994) Hepatology 19:1521.) In addition to growth factor therapy, liver cell growth can also be stimulated by nutritional minipulation. A variety of different nutritional regimines can be used. For instance, a period of protein deprivation followed by consumption of a high protein meal can be used to stimulate DNA synthesis and cell division. (See Mead, et al. (1990) Cancer Res. 50:7023-7030.)

Another example of stimulation of cell division in a particular tissue is the use of cyclooxidase inhibitors, such as indomethacin, to induce hyperplasia in the gastric mucosa. In particular, indomethacin is known to increase DNA synthesis and cell clearance in duodenal and jejunal mucusa (see Uribe, et al. (1992) Dig. Dis. Sci. 37:403-408). Thus, pretreatment with indomethacin or other non-steroidal anti inflammatory drugs can be used to increase cell proliferation in the gastric mucosa. In addition prostaglandins, in particular, prostraglandin E2 can be used after introduction of the gene therapy vehicle in order to increase the retention of the transduced intestinal cells.

D. CELL CULTURE

As noted above, the present invention provides high titer recombinant retroviral preparation suitable for administration to humans. In order to produce such high titer preparations, cell culture methods as described below are provided in order to enable the production of high titer recombinant retroviruses. Briefly, a wide variety of methods may be utilized, including for example, the use of fermenters or bioreactors, roller bottles, cell hotels or cell factories, and hollow fiber culture.

In particular, for bioreactors or fermenters, cells are preferably grown on microcarriers (*i.e.*, Cytodex 1 or Cytodex 2; Pharmacia, Piscataway, N.J. at concentrations ranging from 3 to 15 g/L microcarrier. Suitable media, and growth conditions are described by way of a representative illustration in Example 17.

For roller bottles, suitable conditions include those described above for bioreactors, with the exception that microcarrier beads are not utilized. Generally, cells are grown in 850 $cm^2$ roller bottles ("FALCON" Corning, Corning New York) containing DMEM media, along with 15%-20% FBS. Preferably, the bottles are sealed to avoid

contamination, although "open" bottles may also be utilized under appropriate conditions (*e.g.*, 5% $CO_2$). Generally, the roller bottles are incubated at a temperature of 37°C, with a rotation speed of 0.5 rpm/minute.

Cell factories may also be utilized for the large scale cell culture and production of recombinant retroviruses. Briefly, cell factories (also termed 'cell hotels') typically contain 2, 10, or 40 trays, are molded from virgin polystyrene, treated to provide a Nuclon D surface, and assembled by sonic welding one to another. Generally, these factories have two port tubes which allow access to the chambers for adding reagents or removing culture fluid. A 10-layer factory provides 6000$cm^2$ of surface area for growing cells, roughly the equivalent of 27 T-225 flasks. Cell factories are available from a variety of manufactureres, including for example Nunc.

Most cell types are capable of producing high titer vector into the media for 3-6 days, allowing for multiple harvests. Each cell type is tested to determine the optimal harvest time after seeding the culture and optimal number of harvest days. Cells are typically initially grown in DMEM supplemented with 2-20% FBS in roller bottles until the required number of cells for seeding a cell factory is obtained. Cells are then seeded into the factories and 2 liters of culture supernatant containing vector is harvested from each day for four days. Fresh DMEM/FBS is used to replenish the cultures.

Within other aspects of the present inventin, hollow fiber culture methods are provided for the production of recombinant retroviruses. Briefly, high titer retroviral production using hollow fiber cultures is based on increasing viral concentration as the cells are being cultured to a high density in a reduced volume of media. Cells are fed nutrients and waste products are diluted using a larger volume of fresh media which circulates through the lumen of numerous capillary fibers. The cells are cultured on the exterior spaces of the capillary fibers in a bioreactor chamber where cell waste products are exchanged for nutrients by diffusion through 30,000 Dalton pores in the capillary fibers. Retroviruses which are produced from the cell lines are too large to pass through the 30,000 Dalton pore membrane, and thus concentrate in the hollow fiber bioreactor along side of the cells. The volume of media being cultured on the cell side is approximately 10 to 100 fold lower then volumes required for equivalent cell densities cultured in tissue culture dishes or flasks. This decrease fold in volume inversely correlates with the fold induction of titer when hollow fiber retroviral titers are compared to tissue culture dishes or flasks. This 10-100 fold induction in titer is seen when an individual retroviral producer cell line is amiable to hollow fiber growth conditions. To achieve maximum cell density, the individual cells must be able to grow in very close proximity and on top of each other. Many cell lines will not grow in this fashion and retroviral packaging cell lines based on these types of cell lines may not achieve 10 fold increases in titer. Cell lines which would grow very well would be non-adherent cell line and

it is believed that a retroviral producer line based on a non-adherent cell line may reach 100 fold increases in titer compared to tissue culture dishes and flasks.

The harvest procedure, in its original design, is a procedure which uses syringes to evacuate and replace culture sups to harvest the produced vector. This syringe procedure has an associated high risk of possible contamination, which requires a significant number manual connections and disconnection's of media flow paths. However a convenient procedure has been devised which will reduce the risk of contaminating the cultures, increase the daily volumes which can harvested and reduce the time required to handle the culture system. The harvesting procedure is now performed using a batch peristaltic pump drive to deliver precise volumes of fresh media to replace equal volumes of harvest material which is then delivered through thin line tubing into a collection bottle stored at $4^0$C. The pump batch sequence is activated by a timer which can be set at specific time points and the pump can be adjusted to harvest any set volume of harvest material, twenty-four hours a day. The collected supernatant can then be frozen, pooled with earlier harvests, or processed as described elsewhere. This collection procedure can be used for any hollow fiber system including the Cellco (Rockville, Maryland), Unisyn (Tustin, CA), or Cellex (Coon Rapids, MN) systems including ceramic matrix high density culture systems.

## E. CONCENTRATION AND PURIFICATION OF RECOMBINANT RETROVIRAL PARTICLES

As noted above, the present invention provides methods for concentrating and purifying recombinant retroviruses, in order to increase the purity of therapeutic preparation, as well as to increase the titer of recombinant retrovirus that may be given. A wide variety of methods may be utilized for increasing viral concentration and purity, including for example, precipitation of recombinant retroviruses with ammonium sulfate, polyethylene glycol ("PEG") concentration, concentration by centrifugation (either with or without gradients such as PERCOLL, or "cushions" such as sucrose, use of concentration filters (*e.g.*, Amicon filtration), and 2-phase separations. Each of these methods will be discussed in more detail below.

Briefly, in order to accomplish concentration by precipitation of recombinant retroviruses with ammonium sulfate, ammonium sulfate is added slowly to an appropriate concentration, followed by centrifugation and removal of the ammonium sulfate either by dialysis or by separation on a hydrophobic column. One difficulty with this method however, is that in addition to concentration of recombinant retroviruses, other proteinaceous debris may also be concentrated.

Alternatively, recombinant retroviruses may be concentrated from culture medium with PEG (Green et al., *PNAS 67*:385-393, 1970; Syrewicz et al., *Appl. Micro.*

*24*:488-494, 1972). Such methods are rapid, simple, and inexpensive. However, like ammonium sulfate precipitation, use of PEG also concentrates other proteins from solution.

Within other embodiments, recombinant retroviruses may be concentrated by centrifugation, and more particularly, low speed centrifugation. Briefly, low speed centrifugation allows concentration of recombinant retroviruses, while avoiding the difficulties associated with pelleting that accompanies high speed centrifugation (*e.g.*, virus destruction or inactivation). Particularly preferred methods for concentrating viruses by low-speed centrifugation are described below in more detail in Example 15.

Within yet other aspects of the invention, recombinant retroviruses may be concentrated by an aqueous two-phase separation method. Briefly, polymeric aqueous two-phase systems may be prepared by dissolving two different non-compatible polymers in water. Many pairs of water-soluble polymers may be utilized in the construction of such two-phase systems, including for example polyethylene glycol ("PEG") or methylcellulose, and dextran or dextran sulfate (see Walter and Johansson, *Anal. Biochem. 155*:215-242, 1986; Albertsson, "Partition of Cell Particles and Macromolecules" Wiley, New York, 1960). As described in more detail below in Example 13, utilizing PEG at concentrations ranging from 5% to 8% (preferably 6.5%), and dextran sulfate at concentrations ranging from 0.4% to 1% (preferably 0.4%), an aqueous two-phase system may be established suitable for purifying recombinant retroviruses. Utilizing such procedures, approximately 1.4 liters of crude research grade supernatant may be reduced to a 10 mL volume, while recovering approximately 50% of the total starting retrovirus.

For purposes of illustration, one representative concentration process which combines several concentration steps is set forth below. Briefly, recombinant retroviruses may be prepared either from roller bottles, cell factories, or bioreactors prior to concentration. Preferably, daily harvests of recombinant retroviruses from producer cells is preferred, followed by addition of fresh media. Removed media containing the recombinant retrovirus may be frozen at -70°C, or more preferably, stored at 2°C to 8°C in large pooled batches prior to processing.

For material obtained from a bioreactor, the recombinant retrovirus pool is first clarified through a 0.8 um filter (1.2um glass fiber pre-filter, 0.8um cellulose acetate) connected in series with a 0.65 um filter (Sartorious). This filter arrangement provides approximately 2 square feet of filter, and allows processing of about 15-20 liters of pooled material before clogging. For material obtained from roller bottles or cell factories, a single 0.65um cartridge (2 sq. ft.) normally suffices for volumes up to 40 liters. For 80 liter cell factory processes, a 5 sq. ft. filter may be required.

Preferably, after clarification, the filter is rinsed with buffer (150 mM NaCl, 25 mM Tris, pH 7.2-7.5). This step has allowed recoveries of recombinant retrovirus ranging from 80% to 120%.

Following clarification, recombinant retroviruses are concentrated by tangential flow ultrafiltration utilizing Filtron units and Sigma Screen cassettes with a 300,000 mw cut off. For bioreactor material (containing 12% to 16% FBS), 4 to 5 liters of material may be concentrated per cassette. For roller bottles or cell factories at 12-16% FBS, 5-6 liters of material may be concentrated per cassette. Finally, for cell factories containing 10% FBS, 8 to 9 liters of material may be concentrated per cassette. Utilizing a pressure differential of 2 psi between filtrate (8 psi) and retentate (10 psi), up to 80 liters of material may be concentrated to a volume of less than 500 mL in under two hours. This process also provides a yield of about 80%.

Following the ultrafiltration step, DNAse is added to a concentration of 50 U/mL, and recirculated at a lower pump speed with the filtrate line closed for 30 minutes. If retroviruses have been trapped within a gel layer formed during the ultrafiltration, this step will break down trapped retrovirus, and improve recovery.

Discontinuous diafiltration is then accomplished by addition of 4 liters of additional buffer, and utilizing the same cross differential pressure set forth above. Generally, recovery after this step is approximately 70%.

Concentrated material is then subjected to column chromatography on a Phamacia S-500 HG size exclusion gel, utilizing 50 mM NaCl and 25 mM Tris pH 7.2-7.5 as minimum salt and ionic strength concentrations. Generally, recombinant retroviruses elute off in the first peak.

Tangential flow filtration may once again be utilized in order to further reduce the volume to under 200 mL. Finally, the concentrated material is sterilized by filtration through a 0.2um Millipore filter (PVDF, or Sterivex).

## F. ASSAYS

Within other aspects of the present invention, methods are provided for quantitating retroviral particles utilizing non-denaturing gels (e.g., 4-15% gradient polyacrylamide gels), along with methods for estimating or quantitating the resultant products such as, for example, staining with coomassie blue or silver stain, followed by densitometry scanning. Such methods, while not capable of discriminating between viable and non-viable vector particles, are advantageous because they are relatively simple and quick. One representative example of such methods is set forth below in Example 10 in more detail.

Within other aspects of the present invention, assays are provided for titering recombinant retrovirus in a sample. Typically, such assays may be based upon presence of a selectable marker, or formation of blue colonies. However, within certain embodiments recombinant retroviruses are provided which do not include a gene coding for a selectable marker. Therefore, antibody and PCR assays, the latter of which is described below, may be employed in order to determine retrovirus titer. To use PCR to amplify sequences unique to the recombinant retroviruses described herein, various primers are required. Such primers can readily be designed by those skilled in the art and will depend on the retroviral vector backbone employed and the components thereof, the particular region(s) desired to be amplified, *etc.* Representative examples of particular primer pairs include those specific for LTR sequences, packaging signal sequences or other regions of the retroviral backbone, include primers specific for the nucleic acid molecule (*i.e.*, non-heterologous sequence) of interest.

Briefly, within one embodiment of the invention a PCR titering assay is performed by growing a known number of cells, transduced with a recombinant retrovirus on 6-well plates for at least 16 hr. before harvest. One well per plate is sacrificed for counting. Cells from the other wells are lysed and their contents isolated. DNA is prepared using a QUIAmp DNA isolation kit (QUIAgen, Inc.,Chatsworth, CA). DNAs are resuspended in 5 x $10^6$ cell equivalents/μL per sample.

To calculate titer, a standard curve is generated using DNA isolated from 5 x $10^6$ untransduced HT1080 cells (negative control) and 5 x $10^6$ HT1080 cells transduced with a known vector and having one copy of that vector per cell genome (positive control), such as may be prepared from packaging cell lines transduced with a recombinant retrovirus encoding a selectable marker, *e.g.*, neomycin resistance. The standard curve is generated by combining different amounts of the positive and negative control DNA and amplifying specific sequences therefrom by PCR using primers specific to a particular region of the recombinant retrovirus. A representative group of mixtures for generating a standard curve is:

| Tube | 100% | 75% | 50% | 25% | 10% | 5% | 0% | Blank |
|---|---|---|---|---|---|---|---|---|
| Positive Control (μL) | 50 | 37.5 | 25 | 12.5 | 5 | 2.5 | 0 | 0 |
| Negative Control (μL) | 0 | 12.5 | 25 | 37.5 | 45 | 47.5 | 50 | 0 |
| Distilled water (μL) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 50 |

Five microliters from each tube is placed into one of eight reaction tubes (duplicates are also prepared), with the remainder bring stored at -20°C. Five microliters from each sample DNA preparations are placed into their own reaction tubes in duplicate. PCR reactions (50

μL total volume) are then initiated by adding 45.0 μL of a reaction mix containing the following components per tube to be tested: 24.5 μL water, 5 μL 10X reaction PCR buffer, 4 μL of 25 mM $MgCl_2$, 4 μL dNTPs (containing 2.5 mM of each of dATP, dGTP, dCTP, and dTTP), 5 μL of primer mix (100 ng or each primer), 0.25 μL TaqStart monoclonal antibody (Clontech Laboratories, Inc., Palo Alto, CA), 1.00 μL TaqStart buffer (Clontech Labs, Inc.), and 0.25 μL AmpliTaq DNA polymerase (Perkin-Elmer, Inc., Norwalk, CN). Just prior to aliquoting the reaction mix to the reaction tubes, 1 μL of $\alpha$-$^{32}$P dCTP (250 μCi; 3000 C/mmol, 10 mCi/mL, Amersham Corp., Arlington Heights, IL) is added into the reaction mix. After aliquoting 45.0 μL the reaction mix into each of the reaction tubes, the tubes are capped and placed into a thermocycler. The particular denaturation, annealing, elongation times and temperatures, and number of thermocycles will vary depending on size and nucleotide composition of the primer pair used. 20 - 25 amplification thermocycles are then performed. 5 μL of each reaction is then spotted on DE81 ion exchange chromatography paper (Whatman, Maidstone, England) and air dried for 10 min. The filter is then washed five times, 100 mL per wash, in 50 mM $Na_2PO_4$, pH 7, 200 mM NaCl, after which it is air dried and then sandwiched in Saran Wrap. Quantitation is performed on a PhosphoImager SI (Molecular Dynamics, Sunnyvale, CA). Filters are typically exposed to a phosphor screen, which stores energy from ionizing radiation, for a suitable period, typically about 120 min. After exposure, the phosphor screen is scanned, whereby light is emitted in proportion to the radioactivity on the original filter. The scanning results are then downloaded and plotted on a log scale as cpm (ordinate) versus percent positive control DNA (abscissa). Titers (infectious units/mL) for each sample are calculated by multiplying the number of cells from which DNA was isolated by the percentage (converted to decimal form) determined from the standard curve based on the detected radioactivity, divided by the volume of recombinant retrovirus used to transduce the cells. As will be appreciated by those in the art, other methods of detection, such as colorimetric methods, may also be employed to label the amplified products.

## G. FORMULATION

Within other aspects of the present invention, methods are provided for preserving an infectious recombinant retrovirus, such that the recombinant retrovirus is capable of infecting mammalian cells upon reconstitution (see U.S. Serial No. 08/153,342). Briefly, recombinant retrovirus which has been purified or concentrated as described above may be preserved by first adding a sufficient amount of a formulation buffer to the media containing the recombinant retrovirus, in order to form an aqueous suspension. The formulation buffer is an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. As utilized within the context of the

present invention, a 'buffering compound" or 'buffering component" should be understood to refer to a substance that functions to maintain the aqueous suspension at a desired pH. The aqueous solution may also contain one or more amino acids.

The recombinant retrovirus can also be preserved in a purified form. More specifically, prior to the addition of the formulation buffer, the crude recombinant retrovirus described above may be clarified by passing it through a filter, and then concentrated, such as by a cross flow concentrating system (Filtron Technology Corp., Nortborough, MA). Within one embodiment, DNase is added to the concentrate to digest exogenous DNA. The digest is then diafiltrated to remove excess media components and establish the recombinant retrovirus in a more desirable buffered solution. The diafiltrate is then passed over a Sephadex S-500 gel column and a purified recombinant retrovirus is eluted. A sufficient amount of formulation buffer is added to this eluate to reach a desired final concentration of the constituents (see, *e.g.*, Example 9) and to minimally dilute the recombinant retrovirus, and the aqueous suspension is then stored, preferably at -70°C or immediately dried. As noted above, the formulation buffer is an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

The crude recombinant retrovirus can also be purified by ion exchange column chromatography. This method is described in more detail in U.S. Patent Application Serial No. 08/093,436. In general, the crude recombinant retrovirus is clarified by passing it through a filter, and the filtrate loaded onto a column containing a highly sulfonated cellulose matrix. The recombinant retrovirus is eluted from the column in purified form by using a high salt buffer. The high salt buffer is then exchanged for a more desirable buffer by passing the eluate over a molecular exclusion column. A sufficient amount of formulation buffer is then added, as discussed above, to the purified recombinant retrovirus and the aqueous suspension is either dried immediately or stored, preferably at -70°C.

The aqueous suspension in crude or purified form can be dried by lyophilization or evaporation at ambient temperature. Specifically, lyophilization involves the steps of cooling the aqueous suspension below the glass transition temperature or below the eutectic point temperature of the aqueous suspension, and removing water from the cooled suspension by sublimation to form a lyophilized retrovirus. Briefly, aliquots of the formulated recombinant retrovirus are placed into an Edwards Refrigerated Chamber (3 shelf RC3S unit) attached to a freeze dryer (Supermodulyo 12K). A multistep freeze drying procedure as described by Phillips et al. (Cryobiology 18:414, 1981) is used to lyophilize the formulated recombinant retrovirus, preferably from a temperature of -40°C to -45°C. The resulting composition contains less than 10% water by weight of the lyophilized retrovirus.

Once lyophilized, the recombinant retrovirus is stable and may be stored at -20°C to 25°C, as discussed in more detail below.

Within the evaporative method, water is removed from the aqueous suspension at ambient temperature by evaporation. Within one embodiment, water is removed through spray drying (EP 520,748). Within the spray drying process, the aqueous suspension is delivered into a flow of preheated gas, usually air, whereupon water rapidly evaporates from droplets of the suspension. Spray drying apparatus are available from a number of manufacturers (e.g., Drytec, Ltd., Tonbridge, England; Lab-Plant, Ltd., Huddersfield, England). Once dehydrated, the recombinant retrovirus is stable and may be stored at -20°C to 25°C. Within the methods described herein, the resulting moisture content of the dried or lyophilized retrovirus may be determined through use of a Karl-Fischer apparatus (EM Science Aquastar™ V1B volumetric titrator, Cherry Hill, NJ), or through a gravimetric method.

The aqueous solutions used for formulation, as previously described, are composed of a saccharide, high molecular weight structural additive, a buffering component, and water. The solution may also include one or more amino acids. The combination of these components act to preserve the activity of the recombinant retrovirus upon freezing and lyophilization, or drying through evaporation. Although a preferred saccharide is lactose, other saccharides may be used, such as sucrose, mannitol, glucose, trehalose, inositol, fructose, maltose or galactose. In addition, combinations of saccharides can be used, for example, lactose and mannitol, or sucrose and mannitol. A particularly preferred concentration of lactose is 3%-4% by weight. Preferably, the concentration of the saccharide ranges from 1% to 12% by weight.

The high molecular weight structural additive aids in preventing viral aggregation during freezing and provides structural support in the lyophilized or dried state. Within the context of the present invention, structural additives are considered to be of "high molecular weight" if they are greater than 5000 m.w. A preferred high molecular weight structural additive is human serum albumin. However, other substances may also be used, such as hydroxyethyl-cellulose, hydroxymethyl-cellulose, dextran, cellulose, gelatin, or povidone. A particularly preferred concentration of human serum albumin is 0.1% by weight. Preferably, the concentration of the high molecular weight structural additive ranges from 0.1% to 10% by weight.

The amino acids, if present, function to further preserve viral infectivity upon cooling and thawing of the aqueous suspension. In addition, amino acids function to further preserve viral infectivity during sublimation of the cooled aqueous suspension and while in the lyophilized state. A preferred amino acid is arginine, but other amino acids such as lysine, ornithine, serine, glycine, glutamine, asparagine, glutamic acid or aspartic acid can

also be used. A particularly preferred arginine concentration is 0.1% by weight. Preferably, the amino acid concentration ranges from 0.1% to 10% by weight.

The buffering component acts to buffer the solution by maintaining a relatively constant pH. A variety of buffers may be used, depending on the pH range desired, preferably between 7.0 and 7.8. Suitable buffers include phosphate buffer and citrate buffer. A particularly preferred pH of the recombinant retrovirus formulation is 7.4, and a preferred buffer is tromethamine.

In addition, it is preferable that the aqueous solution contain a neutral salt which is used to adjust the final formulated recombinant retrovirus to an appropriate iso-osmotic salt concentration. Suitable neutral salts include sodium chloride, potassium chloride or magnesium chloride. A preferred salt is sodium chloride.

Aqueous solutions containing the desired concentration of the components described above may be prepared as concentrated stock solutions.

A particularly preferred method of preserving recombinant retroviruses in a lyophilized state for subsequent reconstitution comprises the steps of (a) combining an infectious recombinant retrovirus with an aqueous solution to form an aqueous suspension, the aqueous suspension including 4% by weight of lactose, 0.1% by weight of human serum albumin, 0.03% or less by weight of NaCl, 0.1% by weight of arginine, and an amount of tromethamine buffer effective to provide a pH of the aqueous suspension of approximately 7.4, thereby stabilizing the infectious recombinant retrovirus; (b) cooling the suspension to a temperature of from -40°C to -45°C to form a frozen suspension; and (c) removing water from the frozen suspension by sublimation to form a lyophilized composition having less than 2% water by weight of the lyophilized composition, the composition being capable of infecting mammalian cells upon reconstitution. It is preferred that the recombinant retrovirus be replication defective and suitable for administration into humans upon reconstitution.

As illustrated in Figures 1 and 2, mannitol and lactose lyophilized recombinant retrovirus formulations were assayed for preservation of viral activity under various storage temperatures as a function of time. Similarly, Figure 3 illustrates the results of assays of trehalose recombinant retrovirus formulations for preservation of viral activity under various storage temperatures as a function of time. Figure 4 depicts a comparison of the viral infectivity of frozen formulated recombinant retrovirus (-80°C) as a liquid and the viral infectivity of lyophilized recombinant retrovirus stored at -20°C. Mannitol formulations may lose considerable activity upon lyophilization (5-6 fold), but appear to remain stable subsequent to the lyophilization event. Although not preferable, such a loss is acceptable assuming sufficient amounts of retrovirus are present in the aqueous solution.

It will be evident to those skilled in the art given the disclosure provided herein that it may be preferable to utilize certain saccharides within the aqueous solution

when the lyophilized retrovirus is intended for storage at room temperature. More specifically, it is preferable to utilize disaccharides, such as lactose or trehalose, particularly for storage at room temperature.

The lyophilized or dehydrated retroviruses of the subject invention may be reconstituted using a variety of substances, but are preferably reconstituted using water. In certain instances, dilute salt solutions which bring the final formulation to isotonicity may also be used. In addition, it may be advantageous to use aqueous solutions containing components known to enhance the activity of the reconstituted retrovirus. Such components include cytokines, such as IL-2, polycations, such as protamine sulfate, or other components which enhance the transduction efficiency of the reconstituted retrovirus. Lyophilized or dehydrated recombinant retrovirus may be reconstituted with any convenient volume of water or the reconstituting agents noted above that allow substantial, and preferably total solubilization of the lyophilized or dehydrated sample.

## H. ADMINISTRATION

As noted above, high titer recombinant retroviral particles of the present invention may be administered to a wide variety of locations including, for example, into sites such as the cerebral spinal fluid, bone marrow, joints, arterial endothelial cells, rectum, buccal/sublingual, vagina, the lymph system, to an organ selected from the group consisting of lung, liver, spleen, skin, blood and brain, or to a site selected from the group consisting of tumors and interstitial spaces. Within other embodiments, the recombinant retrovirus may be administered intraocularly, intranasally, sublinually, orally, topically, intravesically, intrathecally, topically, intravenously, intraperitoneally, intracranially, intramuscularly, or subcutaneously. Other representative routes of administration include gastroscopy, ECRP and colonoscopy, which do not require full operating procedures and hospitalization, but may require the presence of medical personnel.

Considerations for administering the compositions of the present invention include the following:

Oral administration is easy and convenient, economical (no sterility required), safe (over dosage can be treated in most cases), and permits controlled release of the active ingredient of the composition (the recombinant retrovirus). Conversely, there may be local irritation such as nausea, vomiting or diarrhea, erratic absorption for poorly soluble drugs, and the recombinant retrovirus will be subject to "first pass effect" by hepatic metabolism and gastric acid and enzymatic degradation. Further, there can be slow onset of action, efficient plasma levels may not be reached, a patient's cooperation is required, and food can affect absorption. Preferred embodiments of the present invention include the oral administration

of recombinant retroviruses that express genes encoding erythropoietin, insulin, GM-CSF cytokines, various polypeptides or peptide hormones, their agonists or antagonists, where these hormones can be derived from tissues such as the pituitary, hypothalamus, kidney, endothelial cells, liver, pancreas, bone, hemopoetic marrow, and adrenal. Such polypeptides can be used for induction of growth, regression of tissue, suppression of immune responses, apoptosis, gene expression, blocking receptor-ligand interaction, immune responses and can be treatment for certain anemias, diabetes, infections, high blood pressure, abnormal blood chemistry or chemistries (*e.g.*, elevated blood cholesterol, deficiency of blood clotting factors, elevated LDL with lowered HDL), levels of Alzheimer associated amaloid protein, bone erosion/calcium deposition, and controlling levels of various metabolites such as steroid hormones, purines, and pyrimidines. Preferably, the recombinant retroviruses are first lyophilized, then filled into capsules and administered.

Buccal/sublingual administration is a convenient method of administration that provides rapid onset of action of the active component(s) of the composition, and avoids first pass metabolism. Thus, there is no gastric acid or enzymatic degradation, and the absorption of recombinant retroviruses is feasible. There is high bioavailability, and virtually immediate cessation of treatment is possible. Conversely, such administration is limited to relatively low dosages (typically about 10-15 mg), and there can be no simultaneous eating, drinking or swallowing. Preferred embodiments of the present invention include the buccal/sublingual administration of recombinant retroviruses that contain genes encoding self and/or foreign MHC, or immune modulators, for the treatment of oral cancer; the treatment of Sjogren's syndrome via the buccal/sublingual administration of such recombinant retroviruses that contain IgA or IgE antisense genes; and, the treatment of gingivitis and periodontitis via the buccal/sublingual administration of IgG or cytokine antisense genes.

Rectal administration provides a negligible first pass metabolism effect (there is a good blood/lymph vessel supply, and absorbed materials drain directly into the inferior vena cava), and the method is suitable of children, patients with emesis, and the unconscious. The method avoids gastric acid and enzymatic degradation, and the ionization of a composition will not change because the rectal fluid has no buffer capacity (pH 6.8; charged compositions absorb best). Conversely, there may be slow, poor or erratic absorption, irritation, degradation by bacterial flora, and there is a small absorption surface (about $0.05m^2$). Further, lipidophilic and water soluble compounds are preferred for absorption by the rectal mucosa, and absorption enhancers (*e.g.*, salts, EDTA, NSAID) may be necessary. Preferred embodiments of the present invention include the rectal administration of recombinant retroviruses that contain genes encoding colon cancer antigens, self and/or foreign MHC, or immune modulators.

Nasal administration avoids first pass metabolism, and gastric acid and enzymatic degradation, and is convenient. In a preferred embodiment, nasal administration is useful for recombinant retrovirus administration wherein the recombinant retrovirus is capable of expressing a polypeptide with properties as described herein. Conversely, such administration can cause local irritation, and absorption can be dependent upon the state of the nasal mucosa.

Pulmonary administration also avoids first pass metabolism, and gastric acid and enzymatic degradation, and is convenient. Further, pulmonary administration permits localized actions that minimize systemic side effects and the dosage required for effectiveness, and there can be rapid onset of action and self-medication. Conversely, at times only a small portion of the administered composition reaches the brochioli/alveoli, there can be local irritation, and overdosing is possible. Further, patient cooperation and understanding is preferred, and the propellant for dosing may have toxic effects. Preferred embodiments of the present invention include the pulmonary administration of recombinant retroviruses that express genes encoding IgA or IgE for the treatment of conditions such as asthma, hay fever, allergic alveolitis or fibrosing alveolitis, the CFTR gene for the treatment of cystic fibrosis, and protease and collagenase inhibitors such as α-1-antitrypsin for the treatment of emphysema. Alternatively, many of the same types of polypeptides or peptides listed above for oral administration may be used..

Ophthalmic administration provides local action, and permit prolonged action where the administration is via inserts. Further, avoids first pass metabolism, and gastric acid and enzymatic degradation, and permits self-administration via the use of eye-drops or contact lens-like inserts. Conversely, the administration is not always efficient, because the administration induces tearing. Preferred embodiments of the present invention include the ophthalmic administration of recombinant retroviruses that express genes encoding IgA or IgE for the treatment of hay fever conjunctivitis or vernal and atomic conjunctivitis; and ophthalmic administration of recombinant retroviruses that contain genes encoding melanoma specific antigens (such as high molecular weight-melanoma associated antigen), self and/or foreign MHC, or immune modulators.

Transdermal administration permits rapid cessation of treatment and prolonged action leading to good compliance. Further, local treatment is possible, and avoids first pass metabolism, and gastric acid and enzymatic degradation. Conversely, such administration may cause local irritation, is particularly susceptible to tolerance development, and is typically not preferred for highly potent compositions. Preferred embodiments of the present invention include the transdermal administration of recombinant retroviruses that express genes encoding IgA or IgE for the treatment of conditions such as atopic dermatitis and other skin allergies; and transdermal administration of recombinant retroviruses encoding

genes encoding melanoma specific antigens (such as high molecular weight-melanoma associated antigen), self and/or foreign MHC, or immune modulators.

Vaginal administration provides local treatment and one preferred route for hormonal administration. Further, such administration avoids first pass metabolism, and gastric acid and enzymatic degradation, and is preferred for administration of compositions wherein the recombinant retroviruses express peptides. Preferred embodiments of the present invention include the vaginal administration of recombinant retroviruses that express genes encoding self and/or foreign MHC, or immune modulators. Other preferred embodiments include the vaginal administration of genes encoding the components of sperm such as histone, flagellin, etc., to promote the production of sperm-specific antibodies and thereby prevent pregnancy. This effect may be reversed, and/or pregnancy in some women may be enhanced, by delivering recombinant retroviruses vectors encoding immunoglobulin antisense genes, which genes interfere with the production of sperm-specific antibodies.

Intravesical administration permits local treatment for urogenital problems, avoiding systemic side effects and avoiding first pass metabolism, and gastric acid and enzymatic degradation. Conversely, the method requires urethral catheterization and requires a highly skilled staff. Preferred embodiments of the present invention include intravesical administration of recombinant retrovirus encoding antitumor genes such as a prodrug activation gene such thymidine kinase or various immunomodulatory molecules such as cytokines.

Endoscopic retrograde cystopancreatography (ERCP) (goes through the mouth; does not require piercing of the skin) takes advantage of extended gastroscopy, and permits selective access to the biliary tract and the pancreatic duct. Conversely, the method requires a highly skilled staff, and is unpleasant for the patient.

Many of the routes of administration described herein (*e.g.*, into the CSF, into bone marrow, into joints, intravenous, intra-arterial, intracranial intramuscular, subcutaneous, into various organs, intra-tumor, into the interstitial spaces, intra-peritoneal, intralymphatic, or into a capillary bed) may be accomplished simply by direct administration using a needle, catheter or related device. In particular,

within certain embodiments of the invention, one or more dosages may be administered directly in the indicated manner: into the cerebral spinal fluid at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; into bone marrow at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; into joint(s) at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; intravenously at dosages greater than or equal to $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; intra-arterially at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; intra-cranially at dosages greater than or equal to $10^9$, $10^{10}$ or $10^{11}$ cfu; intra-muscularly at dosages greater than or equal to $10^{10}$ or $10^{11}$ cfu; intra-

ocularly at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; pulmonarily at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; nasally at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; sub-lingually at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; rectally at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; orally at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; topically at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; vaginally at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; sub-cutaneously at dosages greater than or equal to $10^9$, $10^{10}$ or $10^{11}$ cfu; inter-vesically at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; into an organ such as the lung, liver, spleen, skin, blood or brain at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; intra-tumor at dosages greater than or equal to $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; intra-peritoneally at dosages greater than or equal to $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; into interstitial spaces at dosages greater than or equal to $10^{10}$ or $10^{11}$ cfu; intra-lymphatically at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; into a capillary bed at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu; or intrathecally at dosages greater than or equal to $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ or $10^{11}$ cfu.

Recombinant retrovirus may be delivered to the target from outside of the body (as an outpatient procedure) or as a surgical procedure, where the vector is administered as part of a procedure with other purposes, or as a procedure designed expressly to administer the vector. Other routes and methods for administration include the non-pareneral routes as well as administration via multiple sites.

The following examples are offered by way of illustration, and not by way of limitation.

# EXAMPLES

## EXAMPLE 1

### PREPARATION OF RETROVIRAL VECTOR BACKBONES

A. Preparation of Retroviral Backbones KT-1 and KT-3B

The Moloney murine leukemia virus (MoMLV) 5' long terminal repeat (LTR) EcoR I-EcoR I fragment, including *gag* sequences, from the N2 vector (Armentano *et al.*, *J. Vir. 61*:1647-1650, 1987; Eglitas *et al.*, *Science 230*:1395-1398, 1985) is ligated into the plasmid $SK^+$ (Stratagene, La Jolla, CA). The resulting construct is designated N2R5. The N2R5 construct is mutated by site-directed *in vitro* mutagenesis to change the ATG start codon to ATT preventing gag expression. This mutagenized fragment is 200 base pairs (bp) in length and flanked by Pst I restriction sites. The Pst I-Pst I mutated fragment is purified from the $SK^+$ plasmid and inserted into the Pst I site of N2 MoMLV 5' LTR in plasmid pUC31 to replace the non-mutated 200 bp fragment. The plasmid pUC31 is derived from pUC19 (Stratagene, La Jolla, CA) in which additional restriction sites Xho I, Bgl II, BssH II and Nco I are inserted between the EcoR I and Sac I sites of the polylinker. This construct is designated pUC31/N2R5gM.

A 1.0 kilobase (Kb) MoMLV 3' LTR EcoR I-EcoR I fragment from N2 is cloned into plasmid $SK^+$ resulting in a construct designated $N2R3^-$. A 1.0 Kb Cla I-Hind III fragment is purified from this construct.

The Cla I-Cla I dominant selectable marker gene fragment from pAFVXM retroviral vector (Kriegler *et al.*, *Cell 38*:483, 1984; St. Louis *et al.*, *PNAS 85*:3150-3154,1988), comprising a SV40 early promoter driving expression of the neomycin (neo) phosphotransferase gene, is cloned into the $SK^+$ plasmid. This construct is designated $SK^+$ $SV_2$-*neo* A 1.3 Kb Cla I-BstB I gene fragment is purified from the $SK^+$ $SV_2$-*neo* plasmid.

KT-3B or KT-1 vectors are constructed by a three part ligation in which the Xho I-Cla I fragment containing the gene of interest and the 1.0 Kb MoMLV 3' LTR Cla I-Hind III fragment are inserted into the Xho I-Hind III site of pUC31/N2R5gM plasmid. This gives a vector designated as having the KT-1 backbone. The 1.3 Kb Cla I-BstB I *neo* gene fragment from the pAFVXM retroviral vector is then inserted into the Cla I site of this plasmid in the sense orientation to yield a vector designated as having the KT-3B backbone.

## EXAMPLE 2

### ORAL ADMINISTRATION OF RECOMBINANT RETROVIRUSES EXPRESSING FACTOR VIII

A. Construction of Full-Length and B Domain Deleted Factor VIII cDNA RETROVECTOR™

The following is a description of the construction of several retroviral vectors encoding a full-length factor VIII cDNA. Further discussion is also provided in U.S. Serial No. ____, filed December 30, 1994 [Attorney's Docket No. 930049.438]. Due to the packaging constraints of retroviral vectors and because selection for transduced cells is not a requirement for therapy, a retroviral backbone, *e.g.*, KT-1, lacking a selectable marker gene is employed.

1. Production of Plasmid Vectors Encoding Full-Length Factor VIII

A gene encoding full length factor VIII can be obtained from a variety of sources. One such source is the plasmid pCIS-F8 (*see* EP 0 260 148), which contains a full length factor VIII cDNA whose expression is under the control of a CMV major immediate-early (CMV MIE) promoter and enhancer. The factor VIII cDNA contains approximately 80 bp of 5' untranslated sequence from the factor VIII gene and a 3' untranslated region of about 500 bp. In addition, between the CMV promoter and the factor VIII sequence lies a CMV intron sequence, or "cis" element. The cis element, spanning about 280 bp, comprises a splice donor site from the CMV major immediate-early promoter about 140 bp upstream of a splice acceptor from an immunoglobulin gene.

More specifically, a plasmid, designated pJW-2, encoding a retroviral vector for expressing full length factor VIII is constructed using the KT-1 backbone from pKT-1. Briefly, in order to facilitate directional cloning of the factor VIII cDNA insert into pKT-1, the unique Xho I site is converted to a Not I site by site directed mutagenesis. The resultant plasmid vector is then opened with Not I and Cla I. pCIS-F8 is digested to completion with Cla I and Eag I, for which there are two sites, to release the fragment encoding full length factor VIII. This fragment is then ligated into the Not I/Cla I restricted vector to generate a plasmid designated pJW-2.

2. Construction of a Truncated Factor VII Retrovector (ND-5)

A plasmid vector encoding a truncation of about 80% (approximately 370 bp) of the 3' untranslated region of the factor VIII cDNA, designated pND-5, is constructed in a pKT-1 vector as follows: As described for pJW-2, the pKT-1 vector employed has its Xho I restriction site replaced by that for Not I. The factor VIII insert is generated by digesting pCIS-F8 with Cla I and Xba I, the latter enzyme cutting 5' of the factor VIII stop codon.

The approximately 7 kb fragment containing all but the 3' coding region of the factor VIII gene is then purified. pCIS-F8 is also digested with Xba I and Pst I to release a 121 bp fragment containing the gene's termination codon. This fragment is also purified and then ligated in a three way ligation with the larger fragment encoding the rest of the factor VIII gene and Cla I/Pst I restricted BLUESCRIPT® KS$^+$ plasmid (Stratagene, *supra*) to produce a plasmid designated pND-2.

The unique Sma I site in pND-2 is then changed to a Cla I site by ligating Cla I linkers (New England Biolabs, Beverly, MA) under dilute conditions to the blunt ends created by a Sma I digest. After recircularization and ligation, plasmids containing two Cla I sites are identified and designated pND-3.

The factor VIII sequence in pND-3, bounded by Cla I sites and containing the full length gene with a truncation of much of the 3' untranslated region, is cloned as follows into a plasmid backbone derived from a Not I/Cla I digest of pKT-1 (a pKT-1 derivative by cutting at the Xho I site, blunting with Klenow, and inserting a Not I linker (New England Biolabs)), which yields a 5.2 kb Not I/Cla I fragment. pCIS-F8 is cleaved with Eag I and Eco RV and the resulting fragment of about 4.2 kb, encoding the 5' portion of the full length factor VIII gene, is isolated. pND-3 is digested with Eco RV and Cla I and a 3.1 kb fragment is isolated. The two fragments containing portions of the factor VIII gene are then ligated into the Not I/Cla I digested vector backbone to produce a plasmid designated pND-5.

3. Construction of the B-Domain Deleted Vector

The precursor DNA for the B-deleted FVIII is obtained from Miles Laboratory. This expression vector is designated p25D and has the exact backbone as pCISF8 above. The Hpa I site at the 3' of the FVIII8 cDNA in p25D is modified to Cla-I by oligolinkers. An Acc I to Cla I fragment is clipped out from the modified p25D plasmid. This fragment spans the B-domain deletion and includes the entire 3' two-thirds of the cDNA. An Acc I to Cla I fragment is removed from the Retrovector™ JW-2 above, and replaced with the modified B-domain deleted fragment just described. This is designated B-del-1.

B. Assay for Factor VIII Expression

1. Assay of KT-ND5 Vector Expression by Transient Packaging and Transduction of Murine Cells

Cell lines, L33, (Dennert, USC Comprehensive Cancer Center, Los Angeles, CA, Patek, *et. al.*, *Int. J. of Cancer 24*:624-628, 1979), BC10ME (Patek, *et al.*, Cell Immuno 72:113, 1982, ATCC# TIB85), L33env, and BCenv (L33*env* and BC*env* express

HIV-1 IIIB*env*, Warner et al, AIDS Res. and Human Retrovirus 7:645, 1991), transduced with the KT-ND5 vector, carrying the amphotropic or VSVG envelope protein are examined for the expression of factor VIII. Non-transduced cells are also analyzed for factor VIII expression and compared with KT-ND5 transduced cells to determine the effect of transduction on protein expression.

Murine cell lines, L33-KT-ND5, L33*env*-KT-ND5, L33*env*, L33, BC10ME, BC10ME-KT-ND5, BC*env*, and BC*env*-KT-ND5, are tested for expression of the KT-ND5 molecule. Cells are grown to subconfluent density and the supernatant is removed following centrifugation at 200 xg. The samples are diluted and assayed by the COATEST® Factor VIII assay (KabiVitrum Diagnostica, Molndal, Sweden).

The assay is performed as follows: 100 µl of culture media sample is mixed with 200µl of working buffer provided in the kit. The mixture is incubated at 37°C for 4 - 5 min., after which 100 µL of a 0.025 M $CaCl_2$ stock solution is added, followed by a 5 min. 37°C incubation. 200 µL of the chromogenic reagent (20 mg S-2222, 335 µg synthetic thrombin inhibitor, I-2581, in 10 mL) is then mixed in. After a 5 min. incubation at 37°C, 100 µL of 20% acetic acid or 2% citric acid is added to stop the reaction. Absorbance is then measured against a blank comprising 50 mM Tris, pH 7.3, and 0.2% bovine serum albumin (BSA). A standard curve based on dilutions of normal human plasma (1.0 IU factor VIII/mL) is used and the assays should be performed in plastic tubes. Serum levels of factor VIII in non-hemophilic patients are in the range of 200 ng/mL.

When this assay is used for patient samples, 9 volumes of blood are mixed with one volume of 0.1 M sodium citrate, at a neutral pH, and centrifuged at 2,000 x g for 5 - 20 min. at 20 - 25°C to pellet cells. Due to heat lability of factor VIII, plasma samples should be tested within 30 min. of isolation or stored immediately at -70°C, although as much as 20% of factor VIII activity may be lost during freezing and thawing.

2. <u>Assay of KT-ND5 Vector Expression by Transient Packaging and Transduction of Human Cells</u>

Cell lines transduced with KT-ND5 are examined for expression of factor VIII. Non-transduced cells are analyzed to compare with KT-ND5 transduced cells and determine the effect that transduction has on expression.

Two human cell lines, JY and JY-KT-ND5 are tested for expression of KT-ND5. Suspension cells grown to $10^6$ cells/ml are removed from culture flasks by pipet and pelleted by centrifugation at 200 xg. The supernatant is removed, diluted, and assayed by the Coatest$^R$ Factor VIII assay as described above in Example 2B1.

C. Transient Transfection and Transduction of Packaging Cell Lines HX and DA with the Vector Construct KT-ND5

1. Plasmid DNA Transfection

The packaging cell line, HX (WO92/05266), are seeded at $5.0 \times 10^5$ cells on a 10 cm tissue culture dish on day 1 with Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS). On day 2, the media is replaced with 5.0 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA co-precipitation is performed by mixing 40.0 µl 2.5 M $CaCl_2$, 10 µg plasmid DNA, and deionized $H_2O$ to a total volume of 400 µl. Four hundred microliters of the DNA-$CaCl_2$ solution is added dropwise with constant agitation to 400 µl precipitation buffer (50 mM HEPES-NaOH, pH 7.1; 0.25 M NaCl and 1.5 mM $Na_2HPO_4$-$NaH_2PO_4$). This mixture is incubated at room temperature for 10 minutes. The resultant fine precipitate is added to a culture dish of cells. The cells are incubated with the DNA precipitate overnight at 37°C. On day 3, the media is aspirated and fresh media is added. The supernatant is removed on day 4, passed through a 0.45 µl filter, and stored at -80°C.

Alternatively, 29 2 3 cells (WO 92/05266) ( these are 293 cells expressing gag and pol) are transfected with the vector DNA and the plasmid pMLP-VSVG ( or other VSVG encoding plasmids) to yield VSVG psuedotyped vector particles that are harvested and stored as described above.

2. Packaging Cell Line Transduction

DA (an amphotropic cell line derived from a D17 cell line ATCC No. 183, WO 92/05266) cells are seeded at $5.0 \times 10^5$ cells/10 cm tissue culture dish in 10 ml DMEM and 10% FBS, 4 µg/ml polybrene (Sigma, St. Louis, MO) on day 1. On day 2, 3.0 ml, 1.0 ml and 0.2 ml of the freshly collected virus-containing HX media is added to the cells. The cells are incubated with the virus overnight at 37°C. On day 3, the media is removed and 1.0 ml DMEM, 10% FBS with 800 µg/ml G418 is added to the plate. Only cells that have been transduced with the vector and contain the neomycin selectable marker will survive. A G418 resistant pool is generated over a period of a week. The pool of cells is dilution cloned by removing the cells from the plate and counting the cell suspension, diluting the cells suspension down to 10 cells/ml and adding 0.1 ml to each well (1 cell/well) of a 96 well plate (Corning, Corning, NY). Cells are incubated for 14 days at 37°C, 10% $CO_2$. Twenty-four clones are selected and expanded up to 24 well plates, 6 well plates then 10 cm plates at which time the clones are assayed for expression and the supernatants are collected and assayed for viral titer.

The titer of the individual clones is determined by infection of HT1080 cells, (ATCC No. CCL 121). On day 1, $5.0 \times 10^5$ HT1080 cells are plated on each well of a 6 well microtiter plate in 3.0 ml DMEM, 10% FBS and 4 µg/ml polybrene. On day 2, the

supernatant from each clone is serially diluted 10 fold and used to infect the HT1080 cells in 1.0 ml aliquots. The media is replaced with fresh DMEM, 10% FBS media, and the cells incubated with the vector overnight at 37°C, 10% $CO_2$. On day 3, selection of transduced cells is performed by replacing the media with fresh DMEM, 10% FBS media containing 800 µg/ml G418. Cells are incubated at 37°C, 10% $CO_2$ for 14 days at which time G418 resistant colonies are scored at each dilution to determine the viral titer of each clone as colony forming units(cfu)/ml.

Using these procedures, cell lines are derived that produce greater than or equal to $10^6$ cfu/ml in culture.

The packaging cell line HX is transduced with vector generated from the DA vector producing cell line in the same manner as described for transduction of the DA cells from HX supernatant.

Transduction of the DA or HX cells with vectors lacking a *neo* selectable marker (Example 1) was performed as described above. However, instead of adding G418 to the cells on day 3, the cells are cloned by limiting dilution. Titer is analyzed as described above.

3. Generation of Producer Cell Line via One Packaging Cell Line

In some situations it may be desirable to avoid using more than one cell line in the process of generating producer lines. In this case, DA cells are seeded at $5.0 \times 10^5$ cells on a 10 cm tissue culture dish on day 1 with DMEM and 10% irradiated (2.5 megarads minimum) FBS. On day 2, the media is replaced with 5.0 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA coprecipitation is performed by mixing 60 µl 2.0 M $CaCl_2$, 10 µg MLP-G plasmid, 10 µg KT-ND5 retroviral vector plasmid, and deionized water to a volume of 400 µl. Four hundred microliters of the DNA-$CaCl_2$ solution is added dropwise with constant agitation to 400 µl 2X precipitation buffer (50 mM HEPES-NaOH, pH 7.1, 0.25 M NaCl and 1.5 mM $Na_2HPO_4$-$NaH_2PO_4$). This mixture is incubated at room temperature for 10 minutes. The resultant fine precipitate is added to a culture dish of DA cells plated the previous day. The cells are incubated with the DNA precipitate overnight at 37°C. On day 3, the medium is removed and fresh medium is added. The supernatant containing G-pseudotyped virus is removed on day 4, passed through a 0.45 µl filter and used to infect the DA packaging cell.

DA cells are seeded at $5.0 \times 10^5$ cells on a 10 cm tissue culture dish in 10 ml DMEM and 10% FBS, 4 mg/ml polybrene (Sigma, St. Louis, MO) on day 1. On day 2, 2.0 ml, 1.0 ml or 0.5 ml of the freshly collected and filtered G-pseudotyped virus containing supernatant is added to the cells. The cells are incubated with the virus overnight at 37°C. On day 3 the medium is removed and 10 ml DMEM, 10% irradiated FBS with 800 µg/ml

G418 is added to the plate. Only cells that have been transduced with the vector and contain the *neo* selectable marker will survive. A G418 resistant pool is generated over the period of 1-2 weeks. The pool is tested for expression and then dilution cloned by removing the cells from the plate, counting the cell suspension, diluting the cell suspension down to 10 cells/ml and adding 0.1 ml to each well (1 cell/well) of a 96-well plate. Cells are incubated for 2 weeks at 37°C, 10% $CO_2$ Twenty-four clones are selected and expanded up to 24-well plates, then 6-well plates, and finally 10 cm plates, at which time the clones are assayed for expression and the supernatants are collected and assayed for viral titer as described above.

D. Detection of Replication Competent Retroviruses (RCR)

1. The Extended $S^+L^-$ Assay

The extended $S^+L^-$ assay determines whether replication competent, infectious virus is present in the supernatant of the cell line of interest. The assay is based on the empirical observation that infectious retroviruses generate foci on the indicator cell line $MiCl_1$ (ATCC No. CCL 64.1). The $MiCl_1$ cell line is derived from the Mv1Lu mink cell line (ATCC No. CCL 64) by transduction with Murine Sarcoma Virus (MSV). It is a non-producer, non-transformed, revertant clone containing a replication defective murine sarcoma provirus, $S^+$, but not a replication competent murine leukemia provirus, $L^-$. Infection of $MiCl_1$ cells with replication competent retrovirus "activates" the MSV genome to trigger "transformation" which results in foci formation.

Supernatant is removed from the cell line to be tested for presence of replication competent retrovirus and passed through a 0.45 μ filter to remove any cells. On day 1, Mv1Lu cells are seeded at $1.0 \times 10^5$ cells per well (one well per sample to be tested) of a 6 well plate in 2 ml DMEM, 10% FBS and 8 μg/ml polybrene. Mv1Lu cells are plated in the same manner for positive and negative controls on separate 6 well plates. The cells are incubated overnight at 37°C, 10% $CO_2$. On day 2, 1.0 ml of test supernatant is added to the Mv1Lu cells. The negative control plates are incubated with 1.0 ml of media. The positive control consists of three dilutions (200 focus forming units (ffu), 20 ffu and 2 ffu each in 1.0 ml media) of MA virus (referred to as pAM in Miller *et al., Molec. and Cell Biol. 5*:431, 1985) which is added to the cells in the positive control wells. The cells are incubated overnight. On day 3, the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. The cells are allowed to grow to confluency and are split 1:10 on day 6 and day 10, amplifying any replication competent retrovirus. On day 13, the media on the Mv1Lu cells is aspirated and 2.0 ml DMEM and 10% FBS is added to the cells. In addition, the $MiCl_1$ cells are seeded at $1.0 \times 10^5$ cells per well in 2.0 ml DMEM, 10% FBS and 8 μg/ml polybrene. On day 14, the supernatant from the Mv1Lu cells is transferred to the corresponding well of the $MiCl_1$ cells and incubated overnight at 37°C, 10% $CO_2$. On day

15, the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. On day 21, the cells are examined for focus formation (appearing as clustered, refractile cells that overgrow the monolayer and remain attached) on the monolayer of cells. The test article is determined to be contaminated with replication competent retrovirus if foci appear on the $MiCl_1$ cells. Using these procedures, it can be shown that the HBV core producer cell lines are not contaminated with replication competent retroviruses.

2. Cocultivation of Producer Lines and MdH Marker Rescue Assay

As an alternate method to test for the presence of RCR in a vector-producing cell line, producer cells are cocultivated with an equivalent number of *Mus dunni* (NIH NIAID Bethesda, MD) cells. Small scale cocultivations are performed by mixing of 5.0 x $10^5$ *Mus dunni* cells with 5.0 x $10^5$ producer cells and seeding the mixture into 10 cm plates (10 ml standard culture media/plate, 4 µg/ml polybrene) at day 0. Every 3-4 days the cultures are split at a 1:10 ratio and 5.0 x $10^5$ *Mus dunni* cells are added to each culture plate to effectively dilute out the producer cell line and provide maximum amplifcation of RCR. On day 14, culture supernatants are harvested, passed through a 0.45 µ cellulose-acetate filter, and tested in the MdH marker rescue assay. Large scale cocultivations are performed by seeding a mixture of 1.0 x $10^8$ *Mus dunni* cells and 1.0 x $10^8$ producer cells into a total of twenty T-150 flasks (30 ml standard culture media/flask, 4 µg/ml polybrene). Cultures are split at a ratio of 1:10 on days 3, 6, and 13 and at a ratio of 1:20 on day 9. On day 15, the final supernatants are harvested, filtered and a portion of each is tested in the MdH marker rescue assay.

The MdH marker rescue cell line is cloned from a pool of *Mus dunni* cells transduced with LHL, a retroviral vector encoding the hygromycin B resistance gene (Palmer *et al., PNAS 84*: 1055-1059, 1987). The retroviral vector can be rescued from MdH cells upon infection of the cells with RCR. One ml of test sample is added to a well of a 6-well plate containing $10^5$ MdH cells in 2 ml standard culture medium (DMEM with 10% FBS, 1% 200 mM L-glutamine, 1% non-essential amino acids) containing 4 µg/ml polybrene. Media is replaced after 24 hours with standard culture medium without polybrene. Two days later, the entire volume of MdH culture supernatant is passed through a 0.45 µ cellulose-acetate filter and transferred to a well of a 6-well plate containing 5.0 x $10^4$ *Mus dunni* target cells in 2 ml standard culture medium containing polybrene. After 24 hours, supernatants are replaced with standard culture media containing 250 µg/ml of hygromycin B and subsequently replaced on days 2 and 5 with media containing 200 µg/ml of hygromycin B. Colonies resistant to hygromycin B appear and are visualized on day 9 post-selection, by staining with 0.2% Coomassie blue.

F. Transduction of Human Cells with KT-ND5 Vector Construct

On day one, HT1080 cells are set up at 2 x $10^4$ cells per well in six well tissue culture plates containing 2 mls standard growth media (DME + 10% FBS ). On day two, ND-5 FVIII retroviral vector particles from a confluent vector producing cell line are harvested as a HX-ND-5 clone. They are filtered through .45 µm syringe filters prior to testing the supernatants. (Alternatively the filtered media supernatants may be frozen at 80 in aliquots for later use.) Polybrene is added to each well such that the final concentration is 8 µg per ml. Thirty minutes later, either diluted or undiluted retroviral vector supernatant is added to duplicate wells. Typical volumes and dilutions are 0.5 ml per well and four or more 1:3 serial dilutions in growth media. As a control, two wells are transduced with the same volume of growth media only. On day three, the wells are refeed with 2mls of fresh media and the cells allowed to reach confluence, which may typically be about day four or five. On this day, the cells are again refeed with one ml per well fresh growth media. Twenty four hours later the media is harvested and filtered as above.

G. Expression of Transduced Vector for FVIII

The expression of vector transduced human cells for FVIII is detected by the Coatest$^R$ assay as described above in Example 2B1. Activity is assayed relative to supernatant from the control wells by counting the cells per well from the two control wells and normalizing FVIII expression data per 1x $10^6$ cells per 24 hours.

H. Administration of Vector Construct

1. Animal Administration Protocol

The intestinal epithelium is an attractive site for gene delivery due to its rapidly proliferating tissue mass and the known location of stem cells in the crypts of Lieberkuhn. The deep location of the stem cells in the crypts and the protective role of the mucus gel layer, makes the retrovirus relatively inaccessible to the tissue cells. However, the accessibility of the retroviral vector to these stem cells can be improved in animal models by the *in vivo* mucus removal method of Sandberg, J., *et al.*,( *Human Gene Therapy* 5:3232-329, 1994).

Male Sprague-Dawley rats obtained from Charles River Breeding Laboratories (Portage, MD.) are anesthetized and the cecum is identified upon opening the peritoneal cavity. A 3 cm ileal segment is isolated from the last Peyer's patch in the terminal ileum and ligated at each end. A plastic catheter attached to a syringe is inserted into the segment and two milliliters of the mucolytic agents dithiothreitol and N-acetyl-cysteine is instilled under mild pressure for two minutes, then removed. This procedure is repeated

once again before filling the segment with 0.2 to 2.0 ml of retroviral vector particles at $10^6$ to $10^{10}$ cfu/ml. The ligatures are removed 1 to 4 hours later and the abdominal cavity is sutured. Control animals are instilled with formulation buffer only.

Blood is collected from the tail vein and assayed for factor VIII production by a sandwich ELISA specific for human factor VIII (according to the modified procedure of Zatloukal, K., *et al.*, *PNAS 91*:5148-5152, 1994). The ELISA is based on two monoclonal antibodies directed against human factor VIII (ESH 4 and ESH 8: American Diagnostica). ESH 4 (25 μg/ml in 1.0 M $NaHCO_3$/0.5 M NaCl, pH 9.0) is coupled to the ELISA plates overnight at 4°C, washed with 0.1% Tween 20 in PBS, and blocked with 1% BSA in PBS. The samples are applied in 0.05 M Tris-HCl/1 M NaCl/2% BSA, pH 7.5, over 4 hr at room temperature, the plates are washed, and ESH 8 (2.5 μg/ml in 0.05 M Tris-HCl/1 M NaCl/2% BSA, pH 7.5,) which has been biotinylated with *N*- hydroxysuccinimidobiotin (Pierce, Rockford, IL.) is added for 2 hr at room temperature. The color reaction is performed with peroxidase-conjugated streptavidin (Boehringer Mannheim, Indianapolis, IN.) and o-phenylenediamine dihydrochloride as substrate. The human factor VIII:c standard (from the National Institute for Biological Standards and Control, Hertfordshire, U.K.) and normal rat plasma are used as references.

2. Human Administration Protocol

Lyophilized recombinant retrovirus containing the gene for Factor VIII expression is formulated into an enteric coated tablet or gel capsule according to known methods in the art. These are described in the following patents: US 4,853,230, EP 225,189, AU 9,224,296, AU 9,230,801, and WO 92144,52.

The capsule is administered orally to be targeted to the jejunum. At 1 to 4 days following oral administration of the recombinant retrovirus, expression of Factor VIII is measured in the plasma and blood by the Coatest$^R$ Factor VIII assay as described in Example 2B1.

## EXAMPLE 3

### INTRAVESICAL ADMINISTRATION OF RECOMBINANT RETROVIRUSES EXPRESSING TK

A. Construction of TK Vector Constructs

1. Construction of plasmids containing vector LTR sequences

All of the following retroviral vectors are based on the N2 vector (Keller et al., *Nature 318*:149-154, 1985). Briefly, 5' and 3' Eco RI LTR fragments (2.8 and 1.0 Kb, respectively) (Armentano, *J. Vir. 61*:1647, 1987; Eglitis, *Science 230*:1395, 1985) are

initially subcloned into the Eco RI site of plasmids $SK^+$ (Stratagene, San Diego, CA) and pUC31. pUC31 is a modification of pUC19 (Stratagene, San Diego, CA) carrying additional restriction sites (Xho I, Bgl II, BssH II, and Nco I) between the Eco RI and Sac I sites of the polylinker. Plasmid N2R3+/- is thereby created from ligation of the $SK^+$ plasmid with the 1.0 Kb 3' LTR fragment. The plasmids p31N2R5+/- and p31N2R3+/- are constructed from the ligation of pUC31 with the 2.8 Kb 5' LTR and packaging signal (Y) or the 1.0 Kb 3' LTR fragment, respectively. In each case N2 refers to the vector source, R refers to the fact that the fragment is an Eco RI fragment, 5 and 3 refer to 5' or 3' LTRs, and + or - refers to the orientation of the insert (see Figures 1-6 for examples of LTR subclones).

In one case, a 1.2 Kb Cla I/Eco RI 5' LTR and W fragment from N2 is subcloned into the same sites of an $SK^+$ vector. This vector is designated pN2CR5. In another case, the 5' LTR containing a 6 bp deletion of the splice donor sequence (Yee *et al.*, Cold Spring Harbor, Quantitative Biology, 51:1021, 1986) is subcloned as a 1.8 Kb Eco RI fragment into pUC31. This vector is designated p31N25D[+], Figure 6.

2. Construction of plasmids containing HSVTK

The coding region and transcriptional termination signals of HSV-1 thymidine kinase gene (HSVTK) are isolated as a 1.8 Kb Bgl II/Pvu II fragment from plasmid 322TK (3.5 kb Bam HI fragment of HSV-1 (McKnight *et al.*) cloned into Bam HI of pBR322 (ATCC No. 31344)) and cloned into Bgl II/Sma I-digested pUC31. This construct is designated pUCTK. For constructs which require deletion of the terminator signals, pUCTK is digested with Sma I and Bam HI and the 0.3 Kb fragment containing the $(A)_n$ signal is removed. The remaining coding sequences and sticky-end Bam HI overhang are reconstituted with a double-stranded oligonucleotide made from the following oligomers:

5' GAG AGA TGG GGG AGG CTA ACT GAG 3' (SEQUENCE ID. NO. 1)

5' GAT CCT CAG TTA GCC TCC CCC ATC TCT C 3' (SEQUENCE ID. NO. 2)

The resulting construct is designated pTKD A, Figure 7.

For diagnostic purposes, the oligonucleotides are designed to destroy the Sma I site while maintaining the Ava I site without changing the translated protein.

The plasmid pPrTKDA (Figure 8), which contains the HSVTK promoter and coding sequence (lacking an $(A)_n$ signal), is constructed as follows.

1. pTKD A is linearized with Bgl II treated with alkaline phosphatase, and gel purified.

2. A 0.8 Kg fragment contained the HSVTK transcriptional promoter is isolated as a Bam HI/Bgl II fragment from p322TK.

3. Products from (1) and (2) are ligated, transformed into bacteria, and positive clones are screened for the proper orientation of the promoter region. A resultant clone is designated pPrTKDA (Figure 8).

3. Construction of retroviral provectors expressing HSVTK from a constitutive promoter

The retroviral provectors pTK-1 and pTK-3 are constructed essentially as described below.

1. The 5 Kb Xho I/Hind III 5' LTR and plasmid sequences are isolated from p31N2R5(+) (Figure 1).

2. HSVTK coding sequences lacking transcriptional termination sequences are isolated as a 1.2 Kb Xho I/Bam HI fragment from pTKDA (Figure 2).

3. 3' LTR sequences are isolated as a 1.0 Kb Bam HI/Hind III fragment from pN2R3(-) (Figure 2).

4. The fragments from steps 1-3 are mixed, ligated, transformed into bacteria, and individual clones identified by restriction enzyme analysis. The construct is designated TK-1 (Figure 9).

5. pTK-3 is constructed by linearizing TK-1 with Bam HI, filling in the 5' overhang and blunt-end ligating a 5'-filled Cla I/Cla I fragment containing the bacterial lac UV5 promoter, SV40 early promoter, plus Tn5 $neo^r$ gene obtained from pAFVXM retroviral vector (Krieger *et al.*, *Cell 39*:483, 1984; St. Louis *et al.*, *PNAS 85*:3150, 1988). Kanamycin-resistant clones are isolated and individual clones are screened for the proper orientation by restriction enzyme analysis (Figure 9).

These constructs were used to generate infectious recombinant vector particles in conjunction with a packaging cell line, such as DA as described above.

B. Determination of the Effect of Ganciclovir on Mouse Colon Carcinoma Cells With or Without TK-3 Vector

An experiment was performed to determine whether or not ganciclovir had an effect on CT26 cells (colon tumor 26, Brattain, Baylor College of Medicine, Houston, TX) that were transduced with DA/TK-3. CT26 cells are transduced with G-pseudotyped TK-3 vector. Twenty-four hours after adding the viral supernatant, the CT26 cells are placed under G-418 selection (450 lg/ml). After 10 days incubation, a G-418 selected pool is obtained and designated CT26TK Neo. CT26 TK Neo cells were seeded into six 10 $cm^2$ plates at a density of 2.5 X $10^6$ per plate. As controls, each of two other cell types, CT26 and CT26 beta-*gal* (this cell line was transduced with a retroviral vector carrying the reporter gene β-galactosidase from *E. coli*.), were also seeded into six 10 $cm^2$ plates as controls. Five plates of each cell type were treated twice per day for four consecutive days

with medium containing ganciclovir concentrations of 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml and 6.25 µg/ml. One plate of each cell type was left untreated. Afterwards, the cells were removed from each dish using trypsin-EDTA, resuspended in DMEM with 10% FBS and counted. The data in Figure 10 shows that even the lowest dose of ganciclovir had a dramatic cytotoxic effect on the CT26 TKneo cells. This dose of ganciclovir (6.25 µg/ml) or even the next higher dose (12.5 µg/ml) did not have an effect on either the CT26 or CT26 beta-*gal* cells. However, beginning at a ganciclovir dose of 25 ug/ml, a dose-dependent decrease in cell growth could be seen, although CT26 TK Neo cells were always more sensitive to the drug.

C. <u>Determination of a Ganciclovir Dose For the Treatment of Mice Injected with CT26 TK Neo Cells</u>

In order to test whether *in vivo* transduction of a murine tumor could be used to treat the disease, an experiment was performed to determine the optimal concentration of ganciclovir necessary to eliminate a tumor that was transduced and selected *in vitro* to assure 100% transduction. Twelve groups of 3 mice each are injected with 2 X $10^5$ CT26TK Neo cells. Six groups of mice are injected with these cells intraperitoneally (I.P.) and six groups of mice are injected subcutaneously (S.C.). Two other groups of 3 mice each are injected with 2 X $10^5$ unmodified CT26 cells (as a control) either I.P. or S.C..

Ten days after the injection of the CT26 or CT26TK Neo cells into these groups of mice, several concentrations of ganciclovir treatment are initiated. Each dose regimen consists of 2 daily AM and PM I.P. injections of ganciclovir. The experiment is summarized in Table A below.

**TABLE A**

| Group | Innoculum | Injection Route | Concentration of Ganciclovir (Mg/Kg) |
|---|---|---|---|
| 1 | CT26 I.P. | 0 | |
| 2 | CT26 TKneo | I.P. | 0 |
| 3 | CT26 TKneo | I.P. | 15.63 |
| 4 | CT26 TKneo | I.P. | 31.25 |
| 5 | CT26 TKneo | I.P. | 62.5 |
| 6 | CT26 TKneo | I.P. | 125.0 |
| 7 | CT26 TKneo | I.P. | 250.0 |
| 8 | CT26 TKneo | I.P. | 500.0 |
| 9 | CT26 Subq. | 0 | |
| 10 | CT26 TKneo | Subq. | 0 |
| 11 | CT26 TKneo | Subq. | 15.63 |
| 12 | CT26 TKneo | Subq. | 31.25 |
| 13 | CT26 TKneo | Subq. | 62.5 |

| | | | |
|---|---|---|---|
| 14 | CT26 TKneo | Subq. | 125.0 |
| 15 | CT26 TKneo | Subq. | 250.0 |
| 16 | CT26 TKneo | Subq. | 500.0 |

After 5 days, all of the mice in the 125 mg/Kg, 250 mg/Kg and 500 mg/Kg treated groups were dead due to the toxic effects of ganciclovir. Mice in the 15.63 mg/Kg, 31.25 mg/Kg and 62.5 mg/Kg treated groups were treated for an additional 7 days and were able to tolerate the treatment. Tumor measurements were made for 23 days Figure 11). CT26TK Neo grew only slightly slower than unmodified CT26 in the absence of ganciclovir. Complete tumor regression was seen in the groups of mice treated with the 62.5 mg/Kg regimen. Partial tumor regression was seen in the 31.25 mg/Kg treated groups. Little or no effect was seen in the 15.63 mg/Kg treated groups as compared to the 2 untreated control groups. Even though there was some toxicity observed in the 62.5 mg/Kg groups, it was not life threatening and reversible upon the discontinuation of the treatments so this concentration was used for future studies (Figure 11). After 24 days, the I.P. injected animals were sacrificed and evaluated. As seen in Figures 12 and 13 the optimal concentration for anti-tumor effect was similar whether the tumor was grown I.P. or S.C..

D. Comparison of Cytotoxicity on CT26 and CT26TK Neo In Vivo Tumor Growth

In order to determine whether ganciclovir has an effect on the growth of unmodified CT26 tumor cells *in vivo*, 2 groups of 7 mice are injected S.C. with 2 X $10^5$ unmodified CT26 cells and 2 groups of 7 mice are injected S.C. with 2 X $10^5$ CT26TK Neo cells. Seven days after tumor implantation, one group of CT26 injected mice and one group of CT26TK Neo injected mice are placed on a twice daily (AM and PM) regimen of I.P. ganciclovir at 62.5 mg/Kg. These mice are treated for 12 days or until the CT26TK Neo injected animals have no detectable tumor burden. Tumor growth is monitored over a three week period. Mice injected with CT26 and treated with ganciclovir had tumors that were somewhat smaller than untreated mice injected with CT26, indicating a small HSVTK-independent inhibition of tumor growth (Figure 14). However, a dramatic decrease in tumor burden was observed if, and only if, CT26 TKneo containing mice were treated with ganciclovir (Figure 14).

E. Determination of the Effect of Ganciclovir on AY-27 Rat Carcinoma Cells With or Without the TK-3 Vector

An experiment is performed to determine whether or not ganciclovir has an effect on AY-27 cells that are transduced with DA/TK-3. AY-27 cells are rat carcinoma cells which have been induced by N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide ( Selman, S., *et al.*, *J Urol 136*:141, 1986). The AY-27 cells are transduced with G-pseudotyped TK-3

vector. Twenty-four hours after adding the viral supernatant, the AY-27 cells are placed under G-418 selection (450 μg/ml). After 10 days incubation, a G-418 selected pool is obtained and designated AY-27TK Neo. AY-27TK Neo cells are seeded into six 10 $cm^2$ plates at a density of 2.5 X $10^6$ per plate. As controls, each of two other cell types, AY-27 and AY-27beta-*gal* (this cell line is transduced with a virus carrying the reporter gene β-galactosidase from *E. coli*.), are also seeded into six 10 $cm^2$ plates as controls. Five plates of each cell type are treated twice per day for four consecutive days with medium containing ganciclovir concentrations of 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml and 6.25 μg/ml. One plate of each cell type is left untreated. Afterwards, the cells are removed from each dish using trypsin-EDTA, resuspended in DMEM with 10% FBS and counted. The data generated can be used to determine the concentration which has the most cytotoxic effect o the AY-27, AY-27 beta-*gal*, or AY-27 TK Neo cells.

F. Determination of a Ganciclovir Dose For the Treatment of Rats Injected with AY-27 TK Neo Cells

In order to test whether *in vivo* transduction of a murine tumor could be used to treat the disease, an experiment was performed to determine the optimal concentration of ganciclovir necessary to eliminate a tumor that was transduced and selected *in vitro* to assure 100% transduction. Twelve groups of 3 rats each are injected with 2 X $10^5$ AY-27TK Neo cells. Six groups of rats are injected with these cells intravesically. Six other groups of 3 rats each are injected intravesically with 2 X $10^5$ unmodified AY-27 cells (as a control).

Ten days after the injection of the AY-27 or AY-27TK Neo cells into these groups of rats, several concentrations of ganciclovir treatment are initiated. Each dose regimen consists of 2 daily AM and PM I.P. injections of ganciclovir. The experiment is summarized in Table B below.

**TABLE B**

| Group | Innoculum | Injection Route | Concentration of Ganciclovir (Mg/Kg) |
|---|---|---|---|
| 1 | AY-27 | | 0 |
| 2 | AY-27 TKneo | intravesically | 0 |
| 3 | AY-27 TKneo | intravesically | 15.63 |
| 4 | AY-27 TKneo | intravesically | 31.25 |
| 5 | AY-27 TKneo | intravesically | 62.5 |
| 6 | AY-27 TKneo | intravesically | 125.0 |
| 7 | AY-27 TKneo | intravesically | 250.0 |
| 8 | AY-27 TKneo | intravesically | 500.0 |

Rats are treated for 12 days, eliminating those that die due to the higher concentrations of ganciclovir. Tumor measurements are made for 20 days, assessing tumor regression in order to determine an optimal ganciclovir concentration.

G. Administration Protocol

1. Rat Administration Protocol

AY-27 rat bladder carcinoma cells are transplanted into the bladders of 20 male Fischer 344 rats ( Charles River Breeding Laboratories, Portage, MD). One to fourteen days following transplantation, tumor-bearing rats weighing between 200 to 300 grams are anesthetized, their bladders are surgically exteriorized and evacuated of urine with a 27-gauge needle. Viral vector particles are instilled into the bladders of 5 rats at $10^6$ to $10^{10}$ in cfu 200 to 2,000 µl of formulation buffer. The addition of 4 to 8 µg/ml of polybrene increases the efficiency of transduction. In order to prevent leakage, the cystotomy is repaired with 7-zero nonabsorbable suture. The virus is allowed to incubate in the presence of the tumor cells for 0.5 to 1.0 hour by keeping the animal anesthetized and thereby preventing voiding. Ten control rats receive 500 µl of formulation buffer only.

Alternatively, 200 to 2,000 µl of vector can be instilled directly into the bladder by catheterization through the urethra following urine evacuation and rinsing with saline.

At 24 to 72 hours after vector treatment, the rats are placed on twice daily (AM and PM) injections of I.P. ganciclovir at the previously determined optimum dose (e.g. 62.5 mg/Kg body weight) for 4 to 12 days. Finally, the rats receive a single daily dose of ganciclovir until the end of the experiment (1 to 10 weeks). Whole bladders are removed and tumor growth is measured.

2. Human Administration Protocol

A urinary (Foley) catheter is inserted through the urethra into the bladder and secured in place. The bladder is evacuated of urine and washed with 100 to 500 mls of sterile saline. Recombinant retroviruses containing the gene for thymidine kinase expression are instilled through the catheter into the bladder at $10^5$ to $10^{11}$ cfu in 10 to 500 ml of formulation buffer preferably containing 4 to 8 µg/ml of polybrene, or other enhancing excipients. The viral particles are allowed to incubate for 0.25 to 12 hours prior to removal of the catheter. After 1 to 7 days, ganciclovir is administered at 1 to 5mg/Kg I.V. (at a constant rate over 1 hour) every 12 hours for 2 to 21 days. The vector can be readministered multiple times (2 to 20), followed by ganciclovir administration. Due to the frequency of granulocytopenia and thrombocytopenia in patients receiving ganciclovir, it is

recommended that neutrophil and platelet counts be performed every two days during the dosing of the drug. Tumor regression is monitored by x-ray and/or biopsy and the treatment repeated if required.

## EXAMPLE 4

### PULMONARY ADMINISTRATION OF RECOMBINANT RETROVIRUS EXPRESSING FACTOR VIII

A. Construction of Full-Length and B Domain Deleted Factor VIII cDNA Retrovector™

The construction of the full-length and B domain deleted Factor VIII retrovectors are described in Example 2A.

B. Aerosolization of Recombinant Retroviruses Expressing Factor VIII

The KT-ND5 viral supernatant in formulation buffer, with 4 to 8 μg/ml of polybrene or other transduction enhancing excipient, is nebulized using a DeVilbiss #15 Atomizer (DeVilbiss Health Care Division, Somerset, PA ) designed to produce 0.3 to 0.5 μm particles (Rousculp, M. , *Human Gene Therapy 3*:471-477, 1992.) The aerosol produced by this nebulizer uses compressed air in a laminar flow hood. The mist is directed into a polypropylene tube, and the condensed vapor, as well as the control viral supernatant, is resterilized by 0.22 μm filtration. Retroviral particles pass through this filter without any significant loss of functional activity.

C. Administration of Vector Construct

1. Rat Administration Protocol

Rats are anesthetized and the trachea is exposed by anterior midline incision. Recombinant retroviral particles expressing the factor VIII gene product are diluted in 300μl of formulation buffer, with or without 4 to 8 μg/ml of polybrene or other transduction enhancing excipient, at $10^5$ to $10^{10}$ cfu/ml and instilled into the trachea though a small gauge needle. Control animals are administered 300μl of formulation buffer only. The incision is sutured and the rats are allowed to recover. Two to fourteen days following viral instillation, blood is drawn from the tail vein and examined for factor VIII production as described in Example 2Hi.

2. Human Administration Protocol

The recombinant retrovirus is administered using a DeVilbiss #15 Atomizer (as described above) for 2 to 60 minutes. Two to fourteen days following administration of the recombinant retoviruses, expression of Factor VIII is measured in the blood and plasma by the Coatest[R] Factor VIII assay ( as described in Example 2B1) .

## EXAMPLE 5

### TRANSDERMAL ADMINISTRATION OF RECOMBINANT RETROVIRUSES

A. Construction of TK-3 Vector Construct

Construction and verification of the TK-3 vector construct and recombinant retroviruses are described in Examples 3A through 3F.

B. Administration of Vector Construct

1. Animal Administration Protocol

Cottontail rabbit papillomavirus (CRPV) provides an animal model for the highly oncogenic human papillomavirus (HPV). Papillomas can be induced in the cottontail rabbit and virus infection leads to three different outcomes in the rabbit (Lin, Y. et el., *J Virol 67*:382, 1993). First, papillomas appear and persist for the lifetime of the rabbit; second, papillomas spontaneously regress 2 to 3 months after infection; and third, papillomas progress to carcinomas after 8 to 15 months.

In this experiment, twelve cottontail rabbits (E. Johnson, Rago, KA) are injected with the B strain of CRPV (Stevens, J. *et al.*, *J Virol 30*:891, 1979) by intradermal injection as described by Stevens, J., *et al.*, (*J Virol 30*:891, 1979). Four to six months after infection, when papillomas form, the animals are divided into two groups. In the first group, papillomas of 6 animals are injected with 25 to 100 µl of formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipient, at $10^5$ to $10^{10}$ cfu/ml though a small gauge needle. In the second group, control animals are administered 25 to 100 µl of formulation buffer only. At 24 to 72 hours after vector treatment, the rabbits are placed on twice daily (AM and PM) injections of I.P. ganciclovir at the previously determined optimum dose (e.g. 62.5 mg/Kg body weight) for 4 to 12 days. Finally, the rabbits receive a single daily dose of ganciclovir until the end of the experiment (1 to 10 weeks). Papilloma regression is visually monitored for 2 to 14 days.

2. Human Administration Protocol

The clinical cutaneous lesions that result from the human papillomavirus (HPV) include common warts, filiform warts, plantar warts, and anogenital warts (reviewed in Cobb, M., *et al. J. Am. Acad. Derm. 22*:547, 1990). In this experiment, patients are divided into two groups. In the first group, 100 to 500 µl of recombinant retrovirus particles at a concentration of $10^9$ cfu/ml in a formulated ointment, preferably containing 4 to 8 µg/ml of polybrene or other enhancing excipients, are applied to the warts using a transdermal delivery system (TDS).

Transdermal delivery systems (TDS) are capable of delivering a drug through intact skin so that it reaches the systemic circulation in sufficient quantity to be therapeutically beneficial. TDS provides a variety of advantages including elimination of gastrointestinal absorption problems and hepatic first pass effect, reduction of dosage and dose intervals, and improved patient compliance. The major components of TDS are a controlled release device composed of polymers, the drug to be administered, excipients, and enhancers, and a fastening system to fix the device to the skin. A number of polymers have been described which include gelatin, gum arabic, paraffin waxes, and cellulose acetate phthalate (Sogibayasi, K., *et al., J. Controlled Release, 29*:177-185, 1994).

The second group receives 100 to 500 µl of vector particle in formulation buffer only. The areas are covered and the viral particles are allowed to incubate for 0.25 to 12 hours prior to removal of the TDS. After 1 to 7 days, ganciclovir is administered at 1 to 5mg/Kg I.V., preferably at a constant rate over 1 hour) every 12 hours for 2 to 21 days. The vector can be readministered multiple times (2 to 20), followed by ganciclovir administration. Due to the frequency of granulocytopenia and thrombocytopenia in patients receiving ganciclovir, it is recommended that neutrophil and platelet counts be performed every two days during the dosing of the drug. The regression of the wart is visually monitored for 2 to 14 days.

## EXAMPLE 6

### OCULAR ADMINISTRATION OF RECOMBINANT RETROVIRUSES FOR E3/19K

A. Cloning of E3/19K Gene into KT-3B

1. Isolation and Purification of Adenovirus

The isolation and purification of adenovirus is described by Green *et al. (Methods in Enzymology 58*: 425, 1979). Specifically, five liters of Hela cells (3-6.0 x $10^5$ cells/ml) are infected with 100-500 plaque forming units (pfu) per ml of adenovirus type 2 (Ad2) virions (ATCC No. VR-846). After incubation at 37°C for 30-40 hours, the cells are placed on ice, harvested by centrifugation at 230 xg for 20 minutes at 4°C, and resuspended

in Tris-HCl buffer (pH 8.1). The pellets are mechanically disrupted by sonication and homogenized in trichlorotrifluoroethane prior to centrifugation at 1,000 xg for 10 min. The upper aqueous layer is removed and layered over 10 mls of CsCl (1.43 g/cm$^3$ ) and centrifuged in a SW27 rotor for 1 hour at 20,000 rpm. The opalescent viral band is removed and adjusted to 1.34 g/cm$^3$ with CsCl and further centrifuged in a Ti 50 rotor for 16-20 hours at 30,000 rpm. The visible viral band in the middle of the gradient is removed and stored at 4°C until purification of adenoviral DNA.

2. Isolation and Purification of Adenovirus DNA

The adenovirus band is incubated with protease for 1 hour at 37°C to digest proteins. After centrifugation at 7,800 xg for 10 minutes at 4°C, the particles are solubilized in 5% SDS at room temperature for 30 minutes before being extracted with equal volumes of phenol. The upper aqueous phase is removed, re-extracted with phenol, extracted three times with ether, and dialyzed in Tris buffer for 24 hours. The viral Ad2 DNA is precipitated in ethanol, washed in ethanol, and resuspended in Tris-EDTA buffer (pH 8.1). Approximately 0.5 mg of viral Ad2 DNA is isolated from virus produced in 1.0 L of cells.

3. Isolation of E3/19K Gene

The viral Ad2 DNA is digested with EcoR I and separated by electrophoresis on a 1% agarose gel. The resulting 2.7 Kb Ad2 EcoR I D fragments, located in the Ad2 coordinate region 75.9 to 83.4, containing the E3/19K gene (Herisse *et al., Nucleic Acids Research 8*:2173, 1980, Cladaras *et al., Virology 140*:28, 1985) are eluted by electrophoresis, phenol extracted, ethanol precipitated, and dissolved in Tris-EDTA (pH 8.1).

4. Cloning of E3/19K Gene into KT-3B

The E3/19K gene is cloned into the EcoR I site of PUC1813. PUC1813 is prepared as essentially described by Kay *et al.* (*Nucleic Acids Research 15*:2778, 1987) and Gray *et al.* (*PNAS 80*:5842, 1983). The E3/19K is retrieved by EcoR I digestion and the isolated fragment is cloned into the EcoR I site of phosphatase-treated pSP73 plasmid. This construct is designated SP-E3/19K. The orientation of the SP-E3/19K cDNA is verified by using appropriate restriction enzyme digestion and DNA sequencing. In the sense orientation, the 5' end of the cDNA is adjacent to the Xho I site of the pSP73 polylinker and the 3' end adjacent to the Cla I site. The Xho I-Cla I fragment containing the E3/19K cDNA in either sense or antisense orientation is retrieved from the SP-E3/19K construct and cloned into the Xho I-Cla I site of the KT-3BB retroviral. This construct is designated KT-3B/E3/19K.

B. Cloning of PCR Amplified E3/19K Gene into KT-3B

1. PCR Amplification of E3/19K Gene

The Ad2 DNA E3/19K gene, including the amino terminal signal sequence, followed by the intraluminal domain and carboxy terminal cytoplasmic tail which allow the E3/19K protein to embed itself in the endoplasmic reticulum (ER), is located between viral nucleotides 28,812 and 29,288. Isolation of the Ad2 E3/19K gene from the viral genomic DNA is accomplished by PCR amplification, with the primer pair shown below:

The forward primer corresponds to the Ad2 nucleotide sequences 28,812 to 28,835. (SEQUENCE ID No. 3)

5'-TATATCTCCAGATGAGGTACATGATTTTAGGCTTG-3'

The reverse primer corresponds to the Ad2 nucleotide sequences 29,241 to 29,213. (SEQUENCE ID No. 4)

5'-TATATATCGATTCAAGGCATTTTCTTTTCATCAATAAAAC-3'

In addition to the Ad2 complementary sequences, both primers contain a five nucleotide "buffer sequence" at their 5' ends for efficient enzyme digestion of the PCT amplicon products. This sequence in the forward primer is followed by the Xho I recognition site and by the Cla I recognition site in the reverse primer. Thus, in the 5' to 3' direction, the E3/19K gene is flanked by Xho I and Cla I recognition sites. Amplification of the E3/19K gene from Ad2 DNA is accomplished with the following PCR cycle protocol:

| Temperature°C | Time (min) | No. Cycles |
|---|---|---|
| 94 | 2.0 | 1 |
| 94 | 0.5 | |
| 55 | 0.17 | 5 |
| 72 | 3.5 | |
| 94 | 0.5 | 30 |
| 70 | 3.5 | |
| 72 | 10.0 | 10 |

2. Ligation of PCR Amplified E3/19K Gene into KT-3B

The E3/19K gene from the SP-E3/19K construct, approximately 780 bp in length, is removed and isolated by 1% agarose/TBE gel electrophoresis. The Xho I-Cla I E3/19K fragment is then ligated into the KT-3B retroviral backbone. This construct is designated KT-3B/E3/19K. It is amplified by transforming *E. coli* , DH5 alpha bacterial strain (Bethesda Research Labs, Gaithersburg, Maryland) with the KT-3B/E3/19K construct. Specifically, the bacteria is transformed with 1-100 ng of ligation reaction mixture DNA. The transformed bacterial cells are plated on LB plates containing ampicillin. The plates are incubated overnight at 37°C, bacterial colonies are selected and DNA prepared from them. The DNA is digested with Xho I and Cla I. The expected endonuclease restriction cleavage fragment sizes for plasmids containing the E3/19K gene are 780 and 1,300 bp.

C. Transduction of Packaging Cell Line DA with the Recombinant Retroviral Vector KT-3B/E3/19K

1. Plasmid DNA Transfection

293 2-3 cells (a cell line derived from 293 cells ATCC No. CRL 1573, (WO 92/05266) 5.0 x $10^5$ cells are seeded at approximately 50% confluence on a 6 cm tissue culture dish. The following day, the media is replaced with 4 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA coprecipitation is performed by mixing 10.0 µg of KT-3B/E3/19K plasmid and 10.0 µg MLP G plasmid with a 2M $CaCl_2$ solution, adding a 1x HEPES buffered saline solution, pH 6.9, and incubating for 15 minutes at room temperature. The calcium phosphate-DNA coprecipitate is transferred to the 293 2-3 cells, which are then incubated overnight at 37°C, 5% $CO_2$. The following morning, the cells are rinsed three times in 1x PBS, pH 7.0. Fresh media is added to the cells, followed by overnight incubation at 37°C, 10% $CO_2$. The following day, the media is collected off the cells and passed through a 0.45 µ filter. This supernatant is used to transduce packaging and tumor cell lines. Transient vector supernatant for other vectors are generated in a similar fashion.

2. Packaging Cell Line Transduction

Packaging cell line transduction is performed as described in Example 2C.

3. Detection Of Replication Competent Retroviruses

Detection of replication competent retroviruses is performed as described in Example 2D

D. Transduction of Cell Lines with the Recombinant Retroviral Vector KT-3B/E3/19K

The following adherent human and murine cell lines are seeded at $5 \times 10^5$ cells/10 cm dish with 4 µg/ml polybrene: HT 1080, Hela, and BC10ME. The following day, 1.0 ml of filtered supernatant from the DA E3/19K pool is added to each of the cell culture plates. The following day, 800 µg/ml G418 is added to the media of all cell cultures. The cultures are maintained until selection is complete and sufficient cell numbers are generated to test for gene expression. The transduced cell lines are designated HT 1080-E3/19K, Hela-E3/19K and BC10ME-E3/19K, respectively.

EBV transformed cell lines (BLCL), and other suspension cell lines, are transduced by co-cultivation with irradiated producer cell line, such as DA-E3/19K. Specifically, irradiated (10,000 rads) producer line cells are plated at $5.0 \times 10^5$ cells/6 cm dish in growth media containing 4 µg/ml polybrene. After the cells have been allowed to attach for 2-24 hours, $10^6$ suspension cells are added. After 2-3 days, the suspension cells are removed, pelleted by centrifugation, resuspended in growth media containing 1mg/ml G418, and seeded in 10 wells of a round bottom 96 well plate. The cultures were expanded to 24 well plates, then to T-25 flasks.

E. Expression of E3/19K in the Recombinant Retroviral Vector Construct KT3B-E3/19K

1. Western Blot Analysis

Radio-immuno precipitation assay (RIPA) lysates are made from selected cultures for analysis of E3/19K expression. RIPA lysates are prepared from confluent plates of cells. Specifically, the media is first aspirated off the cells. Depending upon the size of the culture plate containing the cells, a volume of 100 to 500 ml ice cold RIPA lysis buffer (10 mM Tris, pH 7.4; 1% Nonidet P40; 0.1% SDS; 150 mM NaCl) is added to the cells. Cells are removed from plates using a micropipet and the mixture is transferred to a microfuge tube. The tube is centrifuged for 5 minutes to precipitate cellular debris and the supernatant is transferred to another tube. The supernatants are electrophoresed on a 10% SDS-polyacrylamide gel and the protein bands are transferred to an Immobilon membrane in CAPS buffer (10 mM CAPS (Aldrich, Milwaukee, WI) pH 11.0; 10% methanol) at 10 to 60 volts for 2 to 18 hours. The membrane is transferred from the CAPS buffer to 5% Blotto (5% nonfat dry milk; 50 mM Tris, pH 7.4; 150 mM NaCl; 0.02% Na azide, and 0.05% Tween 20) and probed with a mouse monoclonal antibody to E3/19K (Severinsson *et al., J. Cell. Biol. 101*:540-547, 1985). Antibody binding to the membrane is detected by the use of $^{125}$I-Protein A.

2. FACS Analysis of KT3B-E3/19k-Vector Transduced Cells to Demonstrate Decreased Levels of Class I Expression Compared to Non-Transduced Cells.

Cell lines transduced with the KT3b-E3/19K-vector are examined for MHC class I molecule expression by FACS analysis. Non-transduced cells are also analyzed for MHC class I molecule expression and compared with E3/19K transduced cells to determine the effect of transduction on MHC class I molecule expression.

Murine cell lines, BC10ME, BC10ME-E3/19K, P815 (ATCC No. TIB 64), and P815-E3/19K, are tested for expression of the H-2D$^d$ molecule on the cell surface. Cells grown to subconfluent density are removed from culture dishes by treatment with Versene and washed two times with cold (4°C) PBS plus 1% BSA and 0.02% Na-azide (wash buffer) by centrifugation at 200g. Two million cells are placed in microfuge tubes and pelleted in a microfuge at 200g before removing the supernatant. Cell pellets are resuspended with the H-2D$^d$-specific Mab 34-2-12s (50ml of a 1:100 dilution of purified antibody, ATCC No. HB 87) and incubated for 30 min at 4°C with occasional mixing. Antibody labeled cells are washed two times with 1 ml of wash buffer (4°C) prior to removing the supernatant. Cells are resuspended with a biotinylated goat anti-mouse kappa light chain Mab (50ml, of a 1:100 dilution of purified antibody) (Amersham, Arlington Height, IL) and incubated for 30 min at 4°C. Cells are washed, resuspended with 50ml of avidin conjugated FITC (Pierce, Rockford, IL), and incubated for 30 min at 4°C. The cells are washed once more, resuspended in 1 ml of wash buffer, and held on ice prior to analysis on a FACStar Analyzer (Becton Dickinson, Los Angeles, CA). The mean fluorescence intensity of transduced cells is compared with that of non-transduced cells to determine the effect E3/19K protein has on surface MHC class I molecule expression.

F. Administration of Vector Construct

1. Rat Administration Protocol

Rats are anesthetized and one eye is instilled with 5 to 100 µl of recombinant retroviral particles at a concentration of $10^5$ to $10^{10}$ cfu/ml in formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipient,. Five to one hundred µl of solution containing formulation buffer only is added to the other eye to be used as a control. The solution is allowed to incubate for 1 hour before each eye is rinsed 3 times with 100 µl of saline . Two to seven days following the treatment, the rat is sacrificed, the cornea is removed, and homogenized in 2 ml ice cold RIPA lysis buffer. Expression of E3/19K is detected by Western blot analysis as described in Example 6E1.

2. Human Administration Protocol

Although the rate of corneal transplant rejection is relatively low, the current therapy for those with rejection requires continuous treatment of steroid compounds. This eventually leads to cataract formation, requiring surgery. Therefore, the introduction of a recombinant retrovirus expressing the E3/19K would prevent the need for such a steroid regimen. Ten to five hundred µl of recombinant retroviruses at a concentration of $10^5$ to $10^{10}$ cfu/ml in formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipient in formulation buffer, are administered to the eye of a patient lying in a prone position. The solution is allowed to incubate for 15 to 30 minutes before being washed with saline.

Alternatively, the cornea may be incubated for 1 hour in 1 ml of retroviral vector particles at a concentration of $10^5$ to $10^{10}$ cfu/ml in formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipient, just prior to surgical attachment. In either of the above cases, the progress of the transplant is monitored by visually observing tissue viability.

## EXAMPLE 7

INTRANASAL ADMINISTRATION OF RECOMBINANT RETROVIRUSES EXPRESSING FACTOR VIII

A. Construction of Full-Length and B Domain Deleted Factor VIII cDNA Retrovector™

The construction of the full-length and B domain deleted Factor VIII retrovectors are described in Example 2A

B. Administration of Vector Construct

1. Rat Administration Protocol

The nasal route has been shown to be effective for the administration of a number of molecules due to the extensive network of capillaries located under the nasal mucosa. This facilitates effective systemic absorption and when the drug is administered with absorption promoters, absorption occurs rapidly with high bioavailability (review in Gizurarson, *et al.*, *Acta Pharm* 2:105, 1990).

One group of six Fischer-344 rats are used for nasal administration of the retroviral vector particles for Factor VIII. One to fifty µl of retroviral vector particles at $10^9$ cfu/ml in formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipients are applied with a pipette inserted about 3 to 5 mm into each nostril. Another group is administered formulation buffer without vector in the same manner. Blood

samples are collected from the jugular or tail vein 1 to 14 days later and assayed for factor VIII production as described in Example 2Hi.

2. Human Administration Protocol

Several types of drug delivery devices for the nasal cavity exists (reviewed in Chien, Y. *et al.*, *Crit Rev Therap Drug Carr Sys, 4:*67, 1987). These systems include nasal spray, nose drops, saturated cotton pledget, aerosol spray, and insufflator. The metere-dose nebulizer can deliver a predetermined volume of the formulation to the nasal cavity.

Two groups of patients are used in this study. One group of patients receives 100 to 500 µl of retroviral vector particles at $10^9$ cfu/ml in formulation buffer, with or without 4 to 8 µg/ml of polybrene or other transduction enhancing excipients, applied to each nostril via nasal spray or nasal drops. Another group receives formulation buffer only applied in the same manner. Blood samples are collected 1 to 14 days later and assayed for factor VIII production as described in Example 2B1.

## EXAMPLE 8

### PREPARATION OF RECOMBINANT RETROVIRUS FOR DELIVERY OF HUMAN GROWTH HORMONE

A. Preparation of hGH containing vectors

Vector pDHF828 containing the full-length human growth hormone gene is constructed essentially as follows. Briefly, plasmid pDHF811, was constructed by removing the XhoI- ClaI fragment of the KT-1 retroviral vector described above, and inserting the following oligonucleotide linkers by ligation of the cohesive ends:

Linker sequences:
(SEQUENCE ID# 5) 5' TCGAGGATCC GCCCGGGCGG CCGCATCGAT GTCGACG 3'

(SEQUENCE ID# 6) 5' CGCGTCGA CATCGATGCG GCCGCCCGGG CGGATCC 3'

In particular, the linkers were annealed at 65°C for 20 minutes, 42°C for 20 minutes, 37°C for 20 minutes, and room temperature for 2 hours. The concentrations of both oligonucleotides was 18mM and the salt concentration was 100 mM NaCl. After annealing, 50ml of 1.8 mM annealed linker was digested with ClaI overnight to generate ClaI ends. For ligation, 3nM of KT-1 XhoI - ClaI fragment was mixed with 90nM of linker, and the

resultant mixture incubated at 15°C for 3 hours. The ligated DNA sample was transformed into DH-5α competent cells, followed by screening of transformants.

Plasmid chGH 800 containing the full length cDNA of the hGH gene (Martial, R.A. et al., *Science 205*:602, 1979) was digested with Hind III, blunt-ended with the Klenow fragment enzyme, and cloned into the SrfI site of pDHF811. The resultant plasmid was designated pDHF828.

B. Preparation of hGH expressing recombinant retrovirus

The pDHF828 plasmid was then introduced into the HX packaging cell, using standard procedures and assayed using the HGH Chemiluminescence Kit (HGH 100T) ( Nichols Institute, San Juan Capistrano, CA.), according to a preferred modification of the kit protocol. On day 1, the kit components were warmed to room temperature and gently mixed by inversion before opening any vials. Test samples were centrifuged for 5' at top speed in a microfuge before using them in order to remove fibrin and other debris. All samples were measured in quadruplicate, including the standards. The incubations are performed in 12 x17 polypropylene tubes that have been stored in the dark. One hundred fifty ul of sample or standard were aliquoted into each tube and ul of antibody is added and the samples were mixed gently. One bead was added to each well using the forceps provided in the kit. The tubes were capped, covered with foil, and shaken on an orbital shaker for 24 hr at room temperature. Standards contain 530 pg/ml ( STD D), and serial dilutions were made in zero standard of Std D of 250, 100, 50, 25, 10, 5, and 2.5 pg/ml.

After 24 hours, the tubes were uncapped and 0.5 ml of wash buffer were added. These wash solution was added with enough force to make the bead bounce up off the bottom of the tube. The samples were washed three times with 2.0 ml nanopure water, and aspirated completely each time. The luminometer determinations were done in 12x75 polycarbonate (clear plastic) tubes stored in the dark. The luminometer was pretested with performance control standards.

Usinf this assay, HX/HGH retrovector producing cell lines were generated with titers of $4.8 \times 10^6$ cfu/ml. Introduction of the plasmid into DX packaging cells resulted in production of clonal producer cells with a titer of $1.6 \times 10^7$ cfu/ml.

## EXAMPLE 9

### PRESERVATION OF A RECOMBINANT RETROVIRUS

A. Lactose Formulation of a Recombinant Retrovirus

Crude recombinant retrovirus is obtained from a Celligan bioreactor (New Brunswick, New Brunswick, NJ) containing DA cells transformed with the recombinant retrovirus bound to the beads of the bioreactor matrix. The cells release the recombinant retrovirus into the growth media that is passed over the cells in a continuous flow process. The media exiting the bioreactor is collected and passed initially through a 0.8 micron filter then through a 0.65 micron filter to clarify the crude recombinant retrovirus. The filtrate is concentrated utilizing a cross flow concentrating system (Filtron, Boston, MA). Approximately 50 Units of DNase (Intergen, New York, NY) per ml of concentrate is added to digest exogenous DNA. The digest is diafiltrated using the same cross flow system to 150 mM NaCl, 25 mM tromethamine, pH 7.2. The diafiltrate is loaded onto a Sephadex S-500 gel column (Pharmacia, Piscataway, NJ), equilibrated in 50 mM NaCl, 25 mM tromethamine, pH 7.4. The purified recombinant retrovirus is eluted from the Sephadex S-500 gel column in 50 mM NaCl, 25 mM tromethamine, pH 7.4.

The formulation buffer containing lactose was prepared at a 2X concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 70 mM NaCl, 2 mg/ml arginine, 10 mg/ml HSA, and 100 mg/ml lactose in a final volume of 100 mls at a pH 7.4.

The purified recombinant retrovirus is formulated by adding one part 2X lactose formulation buffer to one part S-500 purified recombinant retrovirus. The formulated recombinant retrovirus can be stored at -70°C to -80°C or dried.

The formulated retrovirus is lyophilized in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer (Edwards High Vacuum, Tonawanda, NY). When the freeze drying cycle is completed, the vials are stoppered under a vacuum following a slight nitrogen gas bleeding. Upon removal, vials are crimped with aluminum seals.

In the given lactose study, formulated liquid product was stored at both -80°C and at -20°C cycling freezer. In Figure 15 viral infectivity of these samples were compared to the viral infectivity of lyophilized samples. The lyophilized samples were stored at -20°C, refrigerator temperature and room temperature. Activity of the samples upon reconstitution are determined by titer assay.

The lyophilized recombinant retrovirus is reconstituted with 1.0 ml water. The infectivity of the reconstituted recombinant retrovirus is determined by a titer activity assay. The assay is conducted on HT 1080 fibroblasts or 3T3 mouse fibroblast cell line (ATCC No. CCL 163). Specifically, $1 \times 10^5$ cells are plated onto 6 cm plates and incubated overnight at 37°C, 10% $CO_2$. Ten microliters of a dilution series of reconstituted recombinant retroviruses are added to the cells in the presence of 4 mg/mL polybrene (Sigma, St. Louis, MO) and incubated overnight at 37°C, 10% $CO_2$. Following incubation,

cells are selected for neomycin resistance in G418 containing media and incubated for 5 days at 37°C, 10% $CO_2$. Following initial selection, the cells are re-fed with fresh media containing G418 and incubated for 5-6 days. After final selection, the cells are stained with Commassie blue for colony detection. The titer of the sample is determined from the number of colonies, the dilution, and the volume used.

Figure 15 demonstrates that storage in lyophilized form at -20°C to refrigerator temperatures retains similar viral activity as a recombinant retrovirus stored in liquid at -80 to -20°C permitting less stringent temperature control during storage.

B. Mannitol Formulation of a Recombinant Retrovirus

The recombinant retrovirus utilized in this example was purified as described in Example 9A.

The formulation buffer containing mannitol was prepared as a 2X concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 35 mM NaCl, 2 mg/ml arginine, 10 mg/ml HSA and 80 mg/ml mannitol at a final volume of 100 mls at a pH 7.4.

The purified recombinant retrovirus is formulated by adding one part mannitol formulation buffer to one part S-500 purified recombinant retrovirus. The formulated recombinant retrovirus can be stored at this stage at -70°C to -80°C or dried.

The formulated retrovirus is dried in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer. When the freeze drying cycle is completed, the vials are stoppered under a vacuum following nitrogen gas bleeding to 700 mbar. Upon removal, vials are crimped with aluminum seals.

In the given mannitol study, formulated liquid product was stored at both -80°C and at -20°C in cycling freezers. The viral infectivity of these samples were compared to the viral infectivity of lyophilized samples, Figure 16 The lyophilized samples were stored at -20°C, refrigerator temperature and room temperature. Activity of the samples upon reconstitution are determined using the titer assay described in Example 9A.

Figure 16 demonstrates that storage in lyophilized form at -20°C to refrigerator temperature retains significant viral activity as compared to recombinant retrovirus stored in liquid at -80°C or -20°C, permitting less stringent temperature control during storage.

C. Trehalose Formulation of a Recombinant Retrovirus

The recombinant retrovirus utilized in this example was purified as described in Example 9A.

The formulation buffer containing trehalose was prepared as a 2X concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 70 mM NaCl, 2.0 mg/ml arginine, 10.0 mg/ml HSA and 100 mg/ml trehalose at a final volume of 100 mls at a pH 7.2.

The purified recombinant retrovirus is formulated by adding one part trehalose formulation buffer to one part S-500 purified recombinant retrovirus. The formulated recombinant retrovirus can be stored at this stage at -70°C to -80°C or dried.

The formulated retrovirus is dried in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer. When the freeze drying cycle is completed, the vials are stoppered under a vacuum following nitrogen gas bleeding to 700 mbar. Upon removal, vials are crimped with aluminum seals.

In the given trehalose study, formulated liquid product was stored at both -80°C and at -20°C in cycling freezers. The viral infectivity of these samples was compared to the viral infectivity of lyophilized samples, Figure 17. The lyophilized samples were stored at -20°C, refrigerator temperature and room temperature. Activity of the samples upon reconstitution are determined using the titer assay as described in Example 9A.

Figure 17 demonstrates that storage in lyophilized form at -20°C to refrigerator temperature retains similar viral activity as compared to recombinant retrovirus stored in liquid at -80°C to -20°C permitting less stringent temperature control during storage.

Viral infectivity of liquid formulated recombinant retrovirus samples stored at -80°C was compared to viral infectivity of lyophilized formulated recombinant retrovirus stored at -20°C. Initially, a bulk of recombinant retrovirus was received and formulated in four different ways as shown below. The formulated recombinant retrovirus was then frozen in bulk for 1.5 months subsequent to being quick thawed and freeze dried. Positive controls were stored at -80°C for comparison with lyophilized samples which were stored at -20°C after freeze-drying. The formulations are listed below:

| Formulation | Sugar Concentration (mg/ml) | Buffer Concentration (mM tromehamine) | Salt Concentration (mM NaCl) | Arginine Concentration (mg/ml) | Human Serum Albumin Concentration (mg/ml) |
|---|---|---|---|---|---|
| Mannitol | 40 | 25 | 25 | 1 | 5 |
| Lactose | 40 | 25 | 75 | 1 | 5 |
| Sucrose | 50 | 25 | 60 | 1 | 5 |
| Trehalose | 50 | 25 | 60 | 1 | 5 |

In the graphs of Figure 18, the y-axis on each of the 4 graphs (A, B, C, D) represent the normalized titer. At an initial time point after lyophilization, t = 0, a titer value was established for both the -80°C liquid sample and the -20°C lyophilized sample. At each time point of the stability study, the titer obtained was divided by the zero time point titer value and the % of original entered onto the graph.

The data demonstrates that post-lyphilization activity is maintained in the lyophilized sample (stored at -20°C) relative to the liquid sample (stored at -80°C). The formulated lyophilized recombinant retrovirus was stored in a -20°C freezer (a frost-free cycling freezer). Comparison to the formulated liquid recombinant retrovirus stored at -80°C indicates the lyophilized form permits less stringent control of storage conditions.

## EXAMPLE 10

### ANALYSIS OF CRUDE AND PURIFIED RECOMBINANT RETROVIRUS

Crude and purified solutions of recombinant retrovirus particles may be separated on gradient polyacrylamide gels utilizing, for example, the PHASTGEL system (Pharmacia Biotech). Briefly, samples are place on 4-15% polyacrylamide gels without pretreatment and electrophoresed for 35 minutes at 250V. The gels are then removed and stained with coomassie blue in order to detect virus and other protein components. The gels are then scanned by laser densitometry in order to determine the content of virus and other components.

Virus bands may be identified by their relative molecular weight and by reverse transcriptase activity (RT). The purpose of this assay is to quantify the activity of reverse transcriptase (RT), an enzyme exclusively associated with all retroviruses. The relative amount of retrovirus in a sample can be determined by measuring the activity of this enzyme in a given preparation.

Briefly, moloney murine leukemia virus reverse transcriptase (Pharmacia, Newark, NJ) is diluted to a concentration of 1 µg/ml by addition of 1x Tris/EDTA buffer solution containing 10 mM Tris-HCl and 1mM EDTA, pH 8.0. One hundred microliters of this solution is added 6.84 ml of sterile $dH_20$, 500 µl of 1M Tris HCl pH 8.0, 10 µl of 0.1M $MnCl_2$, 200 µl of 1M dithiothreitol, 50 µl of 10% Nonidet P40 (NP40), 2 µl of 100 µM dNTP (Pharmacia, Newark, NJ, dNTP Ultrapure Kit™), and 300 µl Methyl - $^3H$ Thymidine 5' - Triphosphate (30-50 Ci/mmol). This mixture is incubated for 1 hour at 37°C in a water bath. Following incubation the sample is placed on ice. Approximately 1.0 ml of 2N HCl is added to the cooled sample. The precipitated radiolabled DNA fragments are vacuum filtered onto glass fiber filters using a Millipore sampling manifold (Millipore,

Philadelphia, PA). The filters are washed, dried, placed in scintillation cocktail, and counted in a Beckman LS5000TD scintillation counter (Beckman, Dallas, TX).

## EXAMPLE 11

### ANALYSIS OF COMPLEMENT RESISTANCE IN VARIOUS PACKAGING CELL LINES

A. Packaging Cell Line Preparation and Transduction

Four different packaging cell lines were used to package pCBß-gal into infectious virions. Two cell lines were derived from D17 dog cells ("D") (ATCC CCL 183), and two were derived from the human embryonic kidney cell line 293 ("2") (ATCC CRL 1573). In the case of both dog and human packaging cells, one cell line expressed the amphotropic envelope from the 4070A virus (*i.e.*, DA and 2A, respectively) and the other expressed the xenotropic envelope from the NZB9-1 virus (*i.e.*, DX and 2X, respectively). All packaging cells were grown in DMEM media (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Salt Lake City, UT).

Each of the four packaging cell lines was transduced with a G-pseudotyped pCBß-gal (*see* Burns et al. (1993), *Proc. Nat'l. Acad. Sci. USA*, *vol.* 90, pp:8033-8037 for a pseudotyping procedure). After G418 selection, the selected cell pools were dilution cloned (except for the amphotropic vector producing human cell line which was maintained as a non-clonal pool). Vector producing cell lines producing the highest titer on HT1080 cells (ATCC No. CCL 121) were selected. Titers were determined approximately 2 days after transduction by G418 colony forming units or by X-gal staining of the monolayers. (*See Current Protocols in Molecular Biology*, Ed. Ausubel et al., for more details on the titering assays.)

Retrovirus containing supernatants were collected from each of the confluent monolayers of the individual amphotropic envelope (DA and 2A) or xenotropic envelope (DX or 2X) vector producing cell lines and filtered through a 0.45 µm filter prior to aliquoting and storage at -70°C.

B. Serum Inactivation Assay

Serum inactivation titer assays were performed as follows: Serum was drawn from at least two different human volunteers, chimpanzees, baboons, and macaque monkeys. Approximately 20-70 mL of blood was collected from each donor into serum separating tubes (Becton Dickinson, Los Angeles, CA). Blood was allowed to clot for 20-30 minutes at room temperature, after which time the samples were centrifuged at 2000 x g for 10

minutes at 4°C. Serum was frozen in approximately 1.1 mL aliquots and stored at -80°C. Vials from each batch were tested for total classical complement activity (Quantiplate, Kallestad Labs, Inc., Chaska, MN), and only batches with "normal" levels of complement activity were used. 1 mL aliquots of fresh, 100% serum were used for each inactivation assay. Complement inactivated controls of each serum were prepared by heat inactivation for 30 minutes at 56°C. Undiluted and diluted preparations of supernatants containing recombinant retroviral particles (BCFU greater than or equal to $10^3$ per mL sera) were mixed 1:10 with medium control, sera, or heat inactivated sera. These mixtures were then incubated at 37°C for 30 minutes. Treated vector particles were then titered by a standard blue colony forming unit assay (*Current Protocols in Molecular Biology, supra*).

The results of this experiment are presented below in Table 1 as BCFU/mL.

**Table 1**

**Packaging Cell Line**

| Serum source | DA | DX | 2A | 2X |
|---|---|---|---|---|
| HUMAN | | | | |
| control | 19,000 | 110,000 | 5,700 | 6,700 |
| 100% sera | 400 | 0 | 6,100 | 7,600 |
| H.I. sera | 18,000 | 100,000 | 10,500 | 8,800 |
| CHIMP | | | | |
| control | 16,200 | 31,600 | 27,800 | 22,400 |
| 100% sera | 900 | 1,700 | 21,500 | 18,300 |
| H.I. sera | 28,300 | 47,000 | 42,700 | 48,500 |
| BABOON | | | | |
| control | 16,000 | 13,000 | 8,000 | 7,200 |
| 100% sera | 0 | 0 | 1,800 | 1,000 |
| H.I. sera | 24,000 | 9,600 | 15,000 | 9,400 |
| MACAQUE | | | | |
| control | 170,000 | 13,000 | 2,500 | 2,300 |
| 100% sera | 0 | 0 | 0 | 10 |
| H.I. sera | 270,000 | 12,000 | 3,000 | 2,200 |

H.I. = heat inactivated
control = DMEM + 10% H.I. fetal bovine serum + Sodium pyruvate
Note: zero's indicate that no blue colonies were detected in 0.1 or 0.2 ml undiluted volumes, therefore indicating that titers were conservatively less than 20-40 BCFU/ml.

Briefly, these results demonstrate that recombinant retroviruses which are made in human packaging cell lines exhibit no detectable sensitivity to inactivation by a heat labile component of human serum, presumably complement, in *in vitro* assays. In contrast, they show partial sensitivity to inactivation by chimp, and then increasing sensitivity to

baboon and macaque serum, in order of increasing phylogenetic distance from man. In addition, these *in vitro* results demonstrate that recombinant retroviruses produced in D17 derived packaging cell lines exhibit near total sensitivity to inactivation by a heat labile component, presumably complement, of human, chimp, baboon and macaque serum.

Other experiments which were conducted employing another recombinant retroviral genome packaged in either of two of the above-described cell lines (dog cell line DA, and human cell line 2A), as well as a different human cell line (HX, a HT1080-derived packaging cell line; confirm that the conclusions drawn from the above results are not vector dependent. Moreover, human producer cell lines derived from 293 and HT1080 generated equally complement resistant vector.

Additional experimental data which was generated using recombinant retroviral particles made in human HT1080-derived packaging cells expressing a different murine envelope tropism, poly, similarly showed insensitivity to heat labile human serum components, further confirming the conclusions above.

## EXAMPLE 12

### RECOMBINANT RETROVIRUS PRODUCTION FROM HOLLOW FIBER CULTURES

A. Culture Initiation

To initiate a hollow fiber culture, first condition the hollow fiber bioreactor (HFB) for 48 hours prior to seeding by simulating a run condition with 100-200 mL of complete growth media at 37°C. The growth media should be what ever the cell line has been adapted to. All liquids in the HFB when originally shipped should be aspirated and replaced with the complete growth media. When seeding the bioreactor, the cells should not have been split more than 48 hours earlier and should be in log growth phase at the time of harvest for the seeding of the HFB. The cells are harvested by trypsinazation and pelleted by centrifugation. The cell pellet is then resuspended in 4 mL of 25% pre-conditioned media and delivered to the extra-capillary space by syringe using the side syringe ports found on the HFB. After seeding the HFB, allow the cells to adhere for 20 to 30 minutes before starting the circulation pump. During this time replace the media used to condition the HFB with 100-200 mL using 25% pre-conditioned media. The circulation feed pump is initiated with the starting flow rate set at 25 mL/min. (setting 5 with 2 long pump pins). After 1 hour from the time of switching the pump on, a one mL sample of media is collected in order to record the initial levels of lactate and ammonia. On a daily schedule, 1 ml samples are collected every 24 hours to assay for the daily production of lactate and ammonia. The initial 100-200 mL of old is exchanged media with fresh media when lactate levels begin to reach 2.0 g/L (or

the equivalent to 22 mM/L) The same volume of media is replaced until the culture approaches daily levels of 20 mmol/L When daily levels of lactate reach 20 mmol/L increase your reservoir bottle size to a 500 mL bottle containing 500 mL of fresh media. The flow feed rate is then increased to 50 mL/min. when the culture begins to produce 2.2 mmol/day of lactate. When daily 500 mL volumes reach 20 mmol/L of lactate, the original Cellco supplied reservoir feeding cap is exchanged for the larger reservoir cap (Unisyn-vender part #240820) adapted for the Cellco system with the addition of tubing and male luer lock fittings. This reservoir cap will accommodate the large 2 Liter Corning bottles. (To avoid the exchange of the reservoir caps during a culture run, initiate the culture run with the larger reservoir cap which can also support smaller bottle sizes.) When daily lactate readings are assayed and recorded, one can calculate the daily levels of lactate production of the culture in order to determine when the culture reaches maximum cell density when the rate of lactate decreases and levels off.

B. <u>Seeding density for the 2X-ß-gal</u>

In order to establish specific seeding requirements, two hollow fiber runs are established. One run is seeded with a low number of cells and one with a high number of cells. Progress of the culture is tracked by analyzing the daily glucose consumption and lactate production levels. Figure 22 is a representative graph of data generated over a two week period of the vector producer cell line 2X-ß-$GAL_{17\text{-}14}$.

In this experiment, one HFB was seeded with $1.3 \times 10^7$ cells (to represent the low seed culture) the other seeded with $1.6 \times 10^8$ cells. In this experiment, the cell line (2x-ß-$GAL_{17\text{-}14}$) was able to initiate a good hollow fiber run under low and high seeding conditions. Being able to incubate the HFB with fewer cells, is only convenient for reducing the effort required for generating the number of cells required to start a culture. However a low seed start also extends the time it takes to reach optimal cell densities which usually yield the highest titers. In this experiment, the cell line used, adapted very well to hollow fiber cultures which eventually required daily media changes of 500 mL per day.

C. <u>Cell Culture Health and Maximum Cell Densities:</u>

In the original Cellco design, it was observed that the original media reservoir cap was not suited to fit larger bottles other than the standard 100 and 500 mL media bottles. This is a problem when aggressive growth cultures require greater than 500 mL daily exchanges of media. Daily multiple changes of media increases the likelihood of culture contamination by increasing the daily handling time of the system. If one does not opt to perform multiple daily exchanges of media then one exposes the culture to daily toxic levels of waste products which can affect the cell expansion of the system along with the length at

which the culture run will survive. Figure 22-B demonstrates lactate concentrations of a culture which required daily 500 mL exchanges of fresh media after reaching day 7 of culture. Figure 22-D is one indication of the health of the culture by tracking the amount of lactate being produced on a daily basis. The graphs indicate that the culture was no longer allowed to expand based on the plateauing of daily production of lactate. Another indicator of the health of the cell culture was the drop in peak titer production which also correlated with the daily exposure of high levels of lactate (See Figure 23). These findings would indicate that optimal titers can be correlated with the maximum cell densities and the relative health of the culture.

D. Optimal Titer Concentrations, Frequency of Harvests and Total Harvest Amounts

ß-gal titers for the above experiment were determined from frozen samples and were titered on 293 cells assayed 48 or 72 hours after transduction. The transduced cells were stained for ß GAL activity and individual cells counted on a hemocytometer giving a titer based on the number of blue cells /mL (BCT/mL). As shown in Figure 23, optimum titers were obtained on day 7 of the high seed culture at 1.8 $x10^8$ BCT/mL from a 72 hour blue cell titer on 293 cells. The duplicate culture initially seeded with a 10 fold lower seeding density, peaked at 5.2 $x10^7$ BCT/mL from a 48 hour blue cell titer. Previous flat stock cultures of 2X b-$GAL_{17-14}$ cultures have been titered using 48 hour blue cell titers on HT1080 cells and have been calculated to be 5 $x10^6$ BCT/mL. If one uses the values obtained from 48 hour blue cell titers, the increase in titer by using hollow fiber systems is ten fold higher than crude supernatants obtained from tissue culture dishes or flasks. These maximum titers were reached prior to hitting the daily 20 mmol/L toxic levels lactate which appeared to reduce the titer produced the following week. Crude supernatants can be harvested every 9 hours with out any loss of titer (See Figure 2). It is predicted that 3 harvests per day can be achieved with minimum loss of titers. In addition, continuous hollow fiber cultures can be maintained for several weeks. When titers were compared between the low and the high seed culture, there was little differences by day 11 between the two seed cultures averaging 4 x $10^7$ BCT/mL.

## EXAMPLE 13

### TWO-PHASE PURIFICATION OF RECOMBINANT RETROVIRUSES

A. Concentration of DA/ND-7 recombinant particles

Five milliliters of formulated DX/ND-7 recombinant retroviral particles at a titer of 3.5 x $10^8$ cfu/ml (total of 1.75 x $10^9$ cfu) is diluted in 1400 ml of media (DMEM

containing 5% Fetal Bovine Serum). Three hundred milliliters of two-phase partitioning components (PEG-8000 (autoclaved), dextran-sulfate, and NaCl) are added to a final concentration of 6.5% PEG, 0.4% dextran-sulphate, and 0.3 M NaCl. The resultant solution is placed into a two-liter separatory funnel, and left in a cold room for 24 hours (including two mixing steps approximately 6 to 16 hours apart).

Following the 24 hour period, the bottom layer (approximately 20 mL) is carefully eluted, and the interphase (approximately 1 mL) is collected in a 15 mL conical FALCON tube. The interphase containing vector is diluted to 10 mL by addition of PBS, and incubated at 37°C in order to bring the solution to room temperature and destabilize the micelles.

To one-half of the diluted interphase, KCl is added to a final concentration 0.4 M, and mixed well. The tube is then placed on ice for ten minutes, and spun for 2 minutes at 2,000 rpm in a bench-top centrifuge. The supernatant is removed and filtered through a 0.45 um syringe filter.

The other half of the interphase containing vector is separated by S-500 Sephadex chromatography in 1X PBS.

The results of these concentration processes, as determined in a BCFU assay, are shown below in Table 1:

TABLE 1

| PHASE | CONCENTRATION |
|---|---|
| Crude | $1.1 \times 10^9$ bcfu |
| Separation: Top phase | $1.4 \times 10^8$ bcfu |
| Separation: Interphase | $7(+/-3) \times 10^8$ bcfu |
| Separation: Bottom phase | $2 \times 10^6$ bcfu |
| Final step: KCl separation | $^*6(+/-3) \times 10^8$ bcfu |
| Final step: S-500 separation | $^*1.8(+/-0.3) \times 10^8$ bcfu |

* Note that since the sample was split into two halves, that these numbers were doubled in order to represent the level of purification that would be expected if the entire 1 mL interphase was separated as indicated.

In summary, 1.4 liters of crude research grade supernatant containing recombinant retroviral particles may be reduced to a 10 mL volume, with approximately 50% (+/-20%) being recovered when KCl separation is utilized as the final step. When S-500

chromatography is utilized as the final step, only about 10% of the initial recombinant retroviral particles are recovered in a 14 mL.

In order to complete concentration of the retroviral vector particles, the vector-containing solution may be further subjected to concentration utilizing an MY- membrane Amicon filter, thereby reducing the volume from 10 to 14 mL, down to less than 1 mL.

## EXAMPLE 14

### PRODUCTION OF VECTOR FROM DX/ND7 βGAL CLONE 87 UTILIZING A CELL FACTORY

DX/ND7 βgal clone 87, an expression vector, was grown in cell factories. Cells were grown in DMEM supplemented with Fetal Bovine Serum in roller bottles until enough cells to seed 20 10-layer cell factories (NUNC) at a 1:3 dilution were obtained. Each 10-layer cell factory is seeded with approximately 0.8 liters of cell medium.

Cells were seeded into the cell factory by pouring media containing cells into the factory so that the suspensions evenly fill the 10 layers. The factory is then carefully tilted away from the port side to prevent the suspension from redistribution in the common tube. Finally, the cell factory is rotated into its final upright position. A hepavent filter is attached to each port. The factory was then placed in a $CO_2$ incubator.

In three days, and for each of the next three days, supernatant containing vector was harvested. The cell factory is placed in a tissue culture hood. One filter is removed and sterile transfer tubing is connected to the open port. The factory is lifted so that supernatant drains into the tubing. Approximately 2 liters of supernatant is harvested from each factory. Fresh DMEM/FBS is used to replenish the lost medium. The transfer tubing is removed and the factory replaced in the incubator. From 20 cell factories, approximately 90 liters of crude vector containing supernatant were obtained.

Verification of the vector was performed by transduction of HT1080 cells. These cells were harvested 2 days later and stained for β-gal protein. The titer of the supernatant was determined to be $2 \times 10^7$/ml.

## EXAMPLE 15

### CONCENTRATION OF RECOMBINANT RETROVIRUS BY LOW-SPEED CENTRIFUGATION

A. Retrovector Supernatant Preparation

Producer cell lines DA/ßgal and HX/DN-7 were cultured in a culture flask and a roller bottle, respectively, containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum plus 1mM L-Glutamine, Sodium pyruvate, non-essential amino acids and antibiotics. Viral supernatant was harvested from the flask and roller bottle, and were filtered through a 0.45 um syringe filter. The filtered supernatants were stored either at 4°C (HX/ND7), or frozen at -70°C (DAß-gal).

B. Virus Concentration

Viral supernatant was aliquoted into 50 ml sterile OAKRIDGE screw cap tubes, and placed into an SS34 rotor for use in a Sorvall centrifuge. The tubes were spun for 1 hour at 16,000 rpm (25,000g-force) at 4°C. Upon completion of the spin, the tubes were removed, the supernatant decanted and a small opaque pellet resuspended in the DMEM media described above.

C. Virus Titration

Concentrated virus was titered on HT1080 cells plated 24 hours earlier at a cell density of $2x10^5$ cells per well in a six well plate + 4 ug/ml polybrene. Briefly, virus preps were diluted from 1/10 to 1/10,000 and 50 ul of each dilution was used to infect one well from the six well plate. Plates were incubated overnight at 37°C. Forty-eight hours later, cells were fixed and stained with X-gal. The results are set forth below in Table 1.

Table 1. Virus Concentration through Low Speed Centrifugation

| | *Experiment number* | | | | |
|---|---|---|---|---|---|
| *Parameter description* | 1 | | 2 | | 3 |
| Virus source | DAß-gal | DAß-gal | HX/ND7 | DAß-gal | HX/ND7 |
| Titer of normal harvest | $4.4 \times 10^6$ | $2.1 \times 10^6$ | $3.2 \times 10^5$ | 5x106 | 5x105 |
| Titer of virus concentrate | $6x10^8$ | $7.4x10^7$ | $3.2x10^7$ | 2.9x108 | 3.9x107 |
| Starting volume | 80 ml | .39 ml | 39 ml | 118ml | 40ml |
| Final concentrate volume | .5 ml | .36 ml | .36 ml | .78ml | .28ml |
| Fold virus concentration | 136X | 34X | 100X | 58X | 78X |
| Virus recovery | 87% | 30% | 91% | 50% | 99% |

As is evident from Table 1, virus recovery ranged from 30% to 99%, with the best recovery being obtained from human producer cells (HX/ND7; recovery ranged from 91% to 99%).

## EXAMPLE 16

### CONCENTRATION OF RECOMBINANT RETROVIRUSES BY ULTRAFILTRATION

S-500 purified supernatant containing the β-gal expressing recombinant retrovirus DX/CB-βgal and partially concentrated supernatant containing the same virus were each filtered through a 0.45 um filter, and loaded into a CENTRIPREP-100 filter (product #4308, Amicon, MA). The supernatants were kept at a temperature of 4 °C throughout this procedure, including during centrifugation. The CENTRIPREP filters were spun three times each for 45 to 60 minutes at 500 x G. Between each spin the filtrate was decanted. The retentate was thus sequentially reduced, such that the initial 15 mL (or 10 mL) volume was reduced to approximately 0.6 mL per unit.

The resultant titer was determined by assaying HT1080 target cells set up at a concentration of $1 \times 10^5$ cells per well 24 hours prior to transduction of the viral sample. Cells were transduced in the presence of 8 ug/ml polybrene and 2 mL growth media (DMEM plus 10% FBS) per well. As shown in Table 1 below, approximately one hundred percent of the virus was recovered utilizing this procedure (note that titers are in BCFU/ml).

Table 1

| | *Pre-centriprep titer/volume.* | *Final titer/volume* |
|---|---|---|
| S-500 | $4 \times 10^7$/15 ml | $1.3 \times 10^9$/0.6 ml |
| *part. conc.* | $3 \times 10^8$/10 ml | $1 \times 10^{10}$/0.6 ml |

## EXAMPLE 17

### PREPARATION OF RECMONBNANT RETROVIRUS IN A BIOREACTOR

A. Freezing protocol

Producer cells are frozen in DMEM media containing 10% to 20% FBS, and 5 to 15% DMSO, at a concentration of $1 \times 10^7$ cells/ml/vial. Cells are frozen in a controlled rate freezer (Series PC, Controlled Rate Freezing System, Custom Biogenic Systems,

Warren MI) at a rate of from 1 to 10 °C per minute. Frozen cells are stored in liquid nitrogen.

B. Bioreactor protocol

Cells are thawed from frozen vials at 37°C, washed once with media to remove DMSO, and expanded into 850 $cm^2$ "FALCON" roller bottles (Corning, Corning, N.Y.) Expanded cell culture is used to inoculate a "CELLIGEN PLUS" bioreactor (5 liter working volume; New Brunswick, Edison, N.J.). The cells are grown on microcarriers (*i.e.*, Cytodex 1 or Cytodex 2; Pharmacia, Piscataway, N.J.) at a concentration of 3 to 15 g/L microcarrier. Initial inoculation densities are from 4 to 9 cells/bead at half to full volume for 2 to 24 hours. The media constituents for virus production are DMEM-high glucose (Irvine Scientific, Santa Ana, CA.) basal media supplemented with FBS (10 to 20%), Glutamine (8 to 15mM), glucose (4.5 to 6.5 g/L), Nonessential amino acids (1X), RPMI 1640 amino acids ( 0.2 to 9.6X), 10 mM HEPES, RPMI 1640 Vitamins (0.2 to 5X).

During culture, pH (6.9 to 7.6) and dissolved oxygen ("DO" 5 to 90%) are controlled by the use of a four gas system which includes air, oxygen, nitrogen, and carbon dioxide. After several days of batch growth the culture is then continuously perfused with fresh media with concurrent continuous harvesting in an escalating perfusion rate of 0.5 to 2.5 volumes/day. Cell retention is the result of differential sedimentation of cell covered beads in a decanting column.

During operation the bioreactor is monitored for viable cells, titer, glucose, lactate, ammonia levels, and lack of contamination. Viable cells and titer range from $1 \times 10^5$ cells/ml to $1 \times 10^7$ cells/ml. Glucose ranges from 6 to 0.25 g/L, Lactate from 1 to 25 mM, and Ammonia ranges from 0.5 to 30 mM. Cells are incubated in the bioreactor for 5 to 25 days

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: CHIRON VIAGENE INC.

(ii) TITLE OF INVENTION: PRODUCTION AND ADMINISTRATION OF HIGH TITER RECOMBINANT RETROVIRUSES

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Chiron Viagene Inc.
(B) STREET: Intellectual Property - P.O. Box 8097
(C) CITY: Emeryville
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94622-8097

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT Unassigned
(B) FILING DATE: Unassigned
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kruse, Norman J.
(B) REGISTRATION NUMBER: 35,235
(C) REFERENCE/DOCKET NUMBER: 1133.500

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (510) 601-3520
(B) TELEFAX: (206) 655-3542

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAGAGATGGG GGAGGCTAAC TGAG 24

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATCCTCAGT TAGCCTCCCC CATCTCTC 28

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TATATCTCCA GATGAGGTAC ATGATTTTAG GCTTG 35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATATATCGA TTCAAGGCAT TTTCTTTTCA TCAATAAAAC 40

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCGAGGATCC GCCCGGGCGG CCGCATCGAT GTCGACG 37

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCGTCGACA TCGATGCGGC CGCCCGGGCG GATCC 35

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 77 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGTGAATTCG AGCTCGGTAC CCGGGGATCC TCTAGAGTCG ACCTGCAGGC ATGCAAGCTT 60

GGCGTAATCA TGGTCAT 77

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala Arg Glu Met Gly Glu Ala Asn
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCGAGAGAT GGGGGAGGCT AACTGAG 27

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGCTCTCTA CCCCCTCCGA TTGACACCTA G 31

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Thr Ile Met Thr Met
1 5

## Claims

WHAT IS CLAIMED

1. A method for obtaining measurable levels of a protein, nucleic acid molecule, or enzymatic product in a bodily fluid or cells of a human, comprising administering to a human a recombinant retroviral preparation having a titer on HT1080 cells of greater than $10^5$ cfu/ml, wherein said recombinant retroviral preparation is capable of directing the expression of a protein, nucleic acid molecule, or enzyme which generates an enzymatic product, such that measurable levels of said protein, nucleic acid molecule, or enzymatic product may be obtained in the bodily fluid or cells of said human.

2. The method according to claim 1 wherein said titer is greater than $10^6$ cfu/ml.

3. The method according to claim 1 wherein said titer is greater than $10^7$ cfu/ml.

4. The method according to claim 1 wherein said titer is greater than $10^8$ cfu/ml.

5. The method according to claim 1 wherein said titer is greater than $10^9$ cfu/ml.

6. The method according to claim 1 wherein said titer is greater than $10^{10}$ cfu/ml.

7. The method according to claim 1 wherein said titer is greater than $10^{11}$ cfu/ml.

8. A method for obtaining measurable levels of a protein, nucleic acid molecule, or enzymatic product in a bodily fluid or cells of a human, comprising administering to a human a recombinant retroviral preparation having a titer in human serum and on HT1080 cells equivalent to its' titer in heat-inactivated serum and on HT1080 cells, wherein said recombinant retroviral preparation is capable of directing the expression of a protein, nucleic acid molecule, or enzyme which generates an enzymatic product, such that measurable levels of said protein, nucleic acid molecule, or enzymatic product may be obtained in the bodily fluid or cells of said human.

9. The method according to claim 1 or 8 wherein said recombinant retrovirus is administered to a site selected from the group consisting of cerebral spinal fluid, bone marrow, joints, arterial endothelial cells, rectum, vagina and the lymph system.

10. The method according to claim 1 or 8 wherein said recombinant retrovirus is administered in a manner selected from the group consisting of intraocularly, intranasally, sublinually, orally, topically, intravesically and intrathecally.

11. The method according to claim 1 or 8 wherein said recombinant retrovirus is administered to an organ selected from the group consisting of lung, liver, spleen, skin, blood and brain.

12. The method according to claim 1 or 8 wherein said recombinant retrovirus is administered intravenously.

13. The method according to claim 1 or 8 wherein said recombinant retrovirus is administered in a manner selected from the group consisting of intracranially, intramuscularly and subcutaneously

14. The method according to claim 1 or 8 wherein said recombinant retrovirus is administered to a site selected from the group consisting of tumors and interstitial spaces.

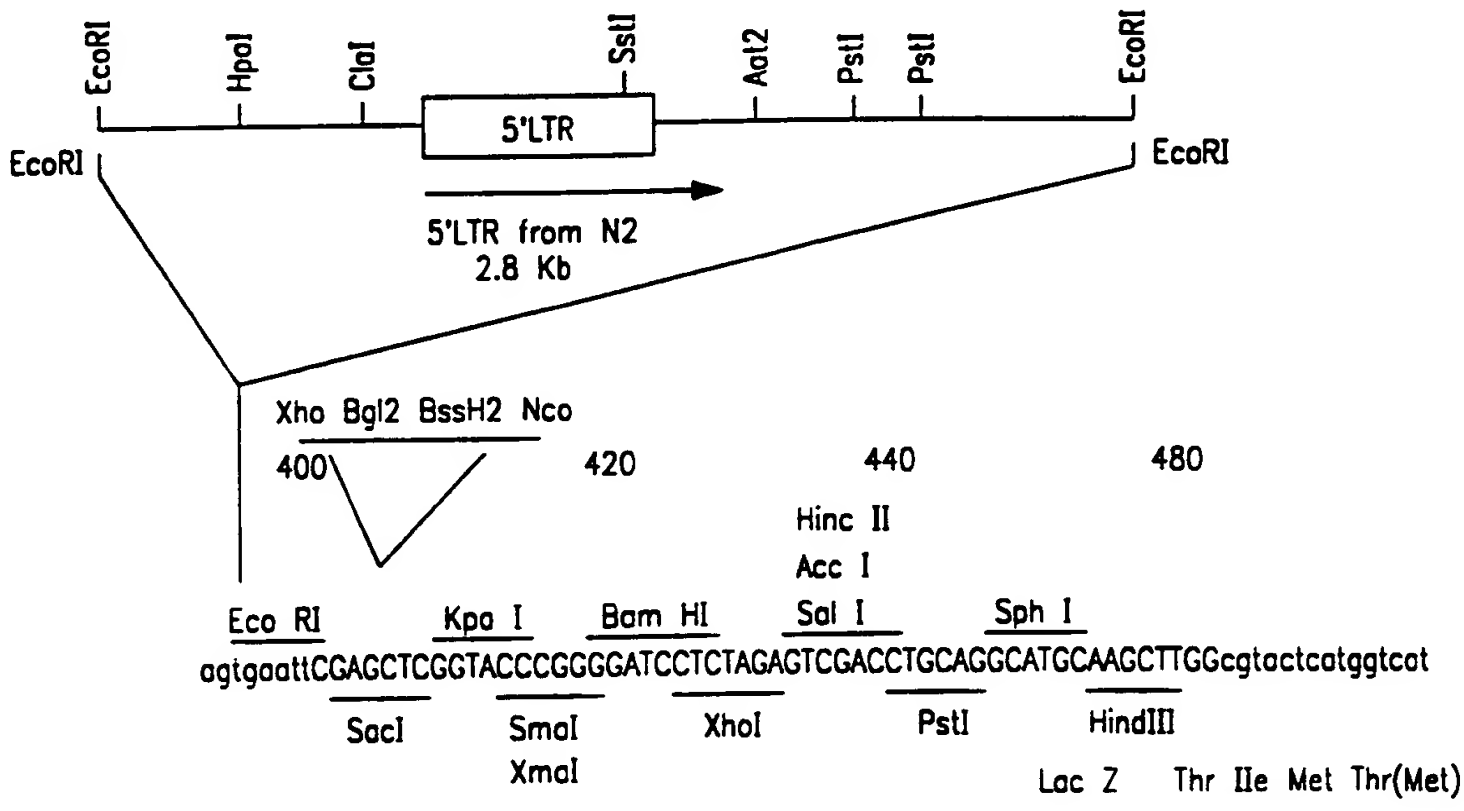
15. The method according to claim 1 or 8 wherein said protein is a viral antigen obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hantavirus, HTLV I, HTLV II and CMV.

16. The method according to claim 1 or 8 wherein said protein is a cytokine selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, γ-IFN, G-CSF and GM-CSF.

17. The method according to claim 1 or 8 wherein said nucleic acid molecule is selected from the group consisting of an antisense sequence, a non-coding non-heterologous sense sequence, and a ribozyme sequence.

18. The method according to claim 1 or 8 wherein said protein is a toxin.

19. The method according to claim 1 or 8 wherein said protein is a Factor VIII.


EcoRI
HpaI
ClaI
SstI
Aat2
PstI
PstI
EcoRI
5'LTR
EcoRI
EcoRI
5'LTR from N2
2.8 Kb
Xho Bgl2 BssH2 Nco
400
420
440
480
Hinc II
Acc I
Eco RI
Kpn I
Bam HI
Sal I
Sph I
agtgaattCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGcgtactcatggtcat
SacI
SmaI
XmaI
XhoI
PstI
HindIII
Lac Z
Thr IIe Met Thr(Met)

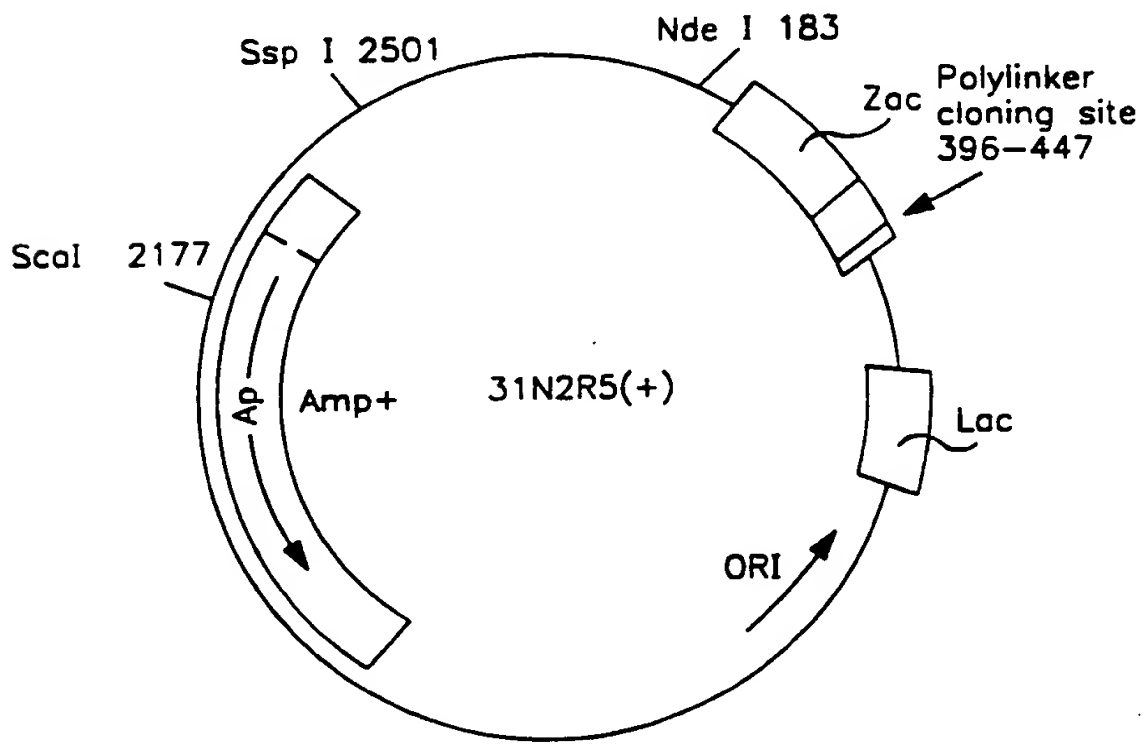

Ssp I 2501
Nde I 183
Zac
Polylinker
cloning site
396–447
ScaI 2177
Ap
Amp+
31N2R5(+)
Lac
ORI

FIG. 1

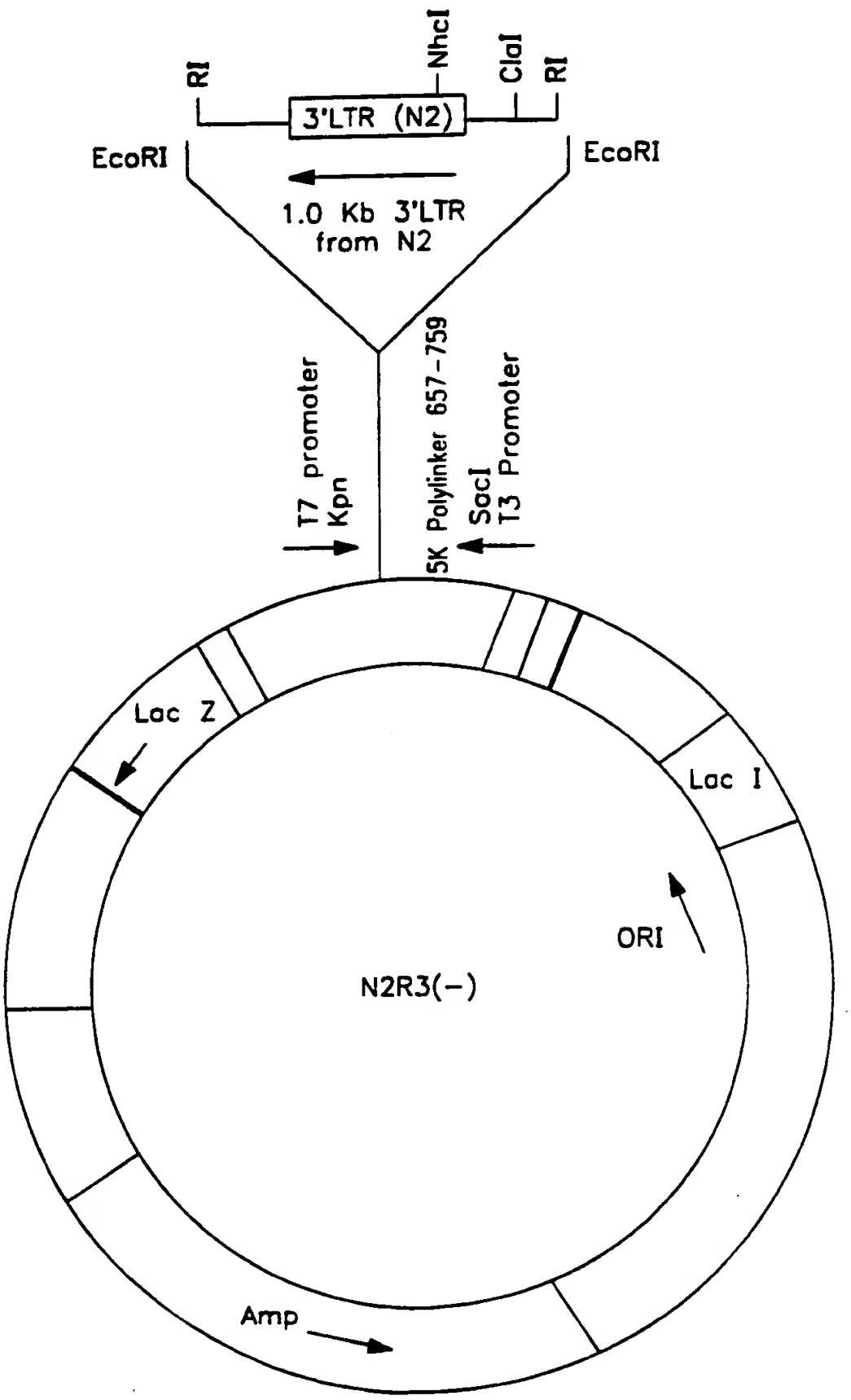
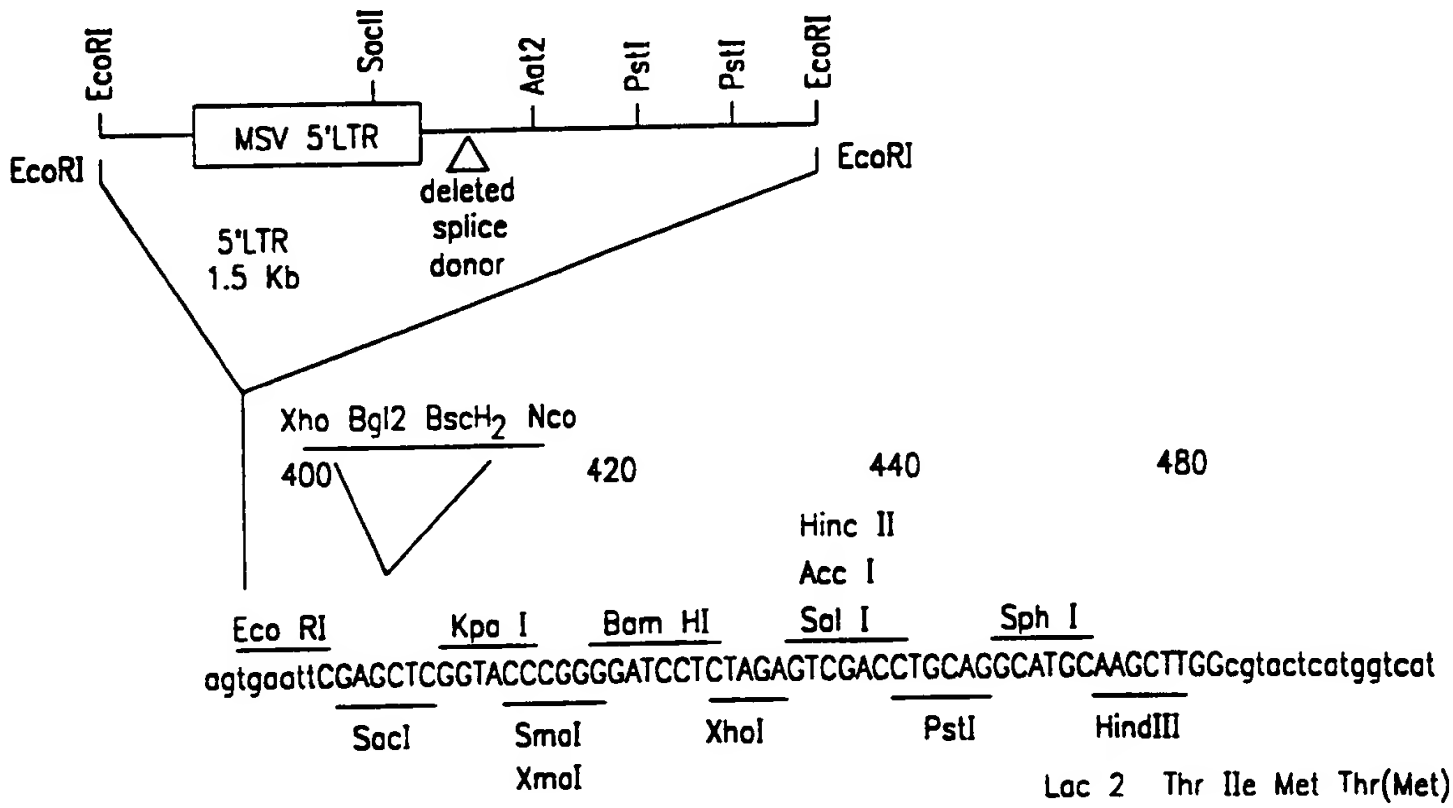

RI
NhcI
ClaI
RI
3'LTR (N2)
EcoRI
EcoRI
1.0 Kb 3'LTR
from N2
T7 promoter
Kpn
5K Polylinker 657-759
SacI
T3 Promoter
Lac Z
Lac I
ORI
N2R3(-)
Amp

FIG. 2


EcoRI
SacII
Aat2
PstI
PstI
EcoRI
MSV 5'LTR
EcoRI
EcoRI
deleted
splice
donor
5'LTR
1.5 Kb
Xho Bgl2 BscH2 Nco
400
420
440
480
Hinc II
Acc I
Eco RI
Kpn I
Bam HI
Sal I
Sph I
agtgaattCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGcgtactcatggtcat
SacI
SmaI
XmaI
XhoI
PstI
HindIII
Lac 2 Thr Ile Met Thr(Met)

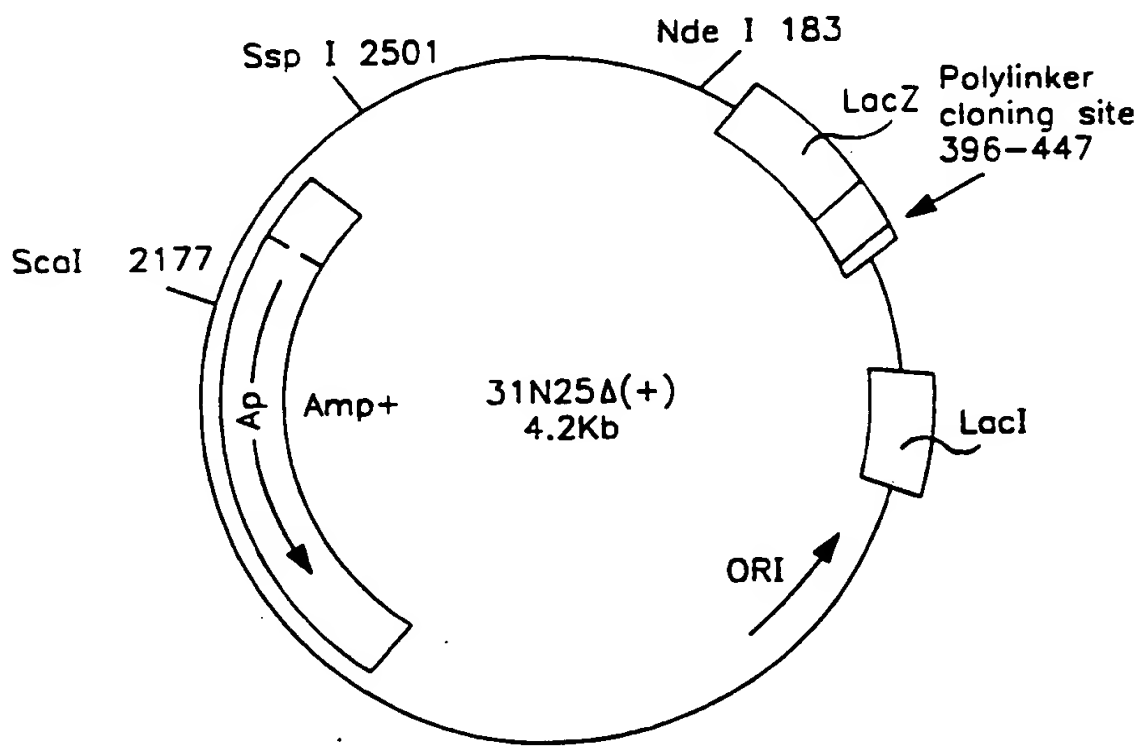

Ssp I 2501
Nde I 183
LacZ
Polylinker
cloning site
396–447
ScaI 2177
Ap
Amp+
31N25Δ(+)
4.2Kb
LacI
ORI

FIG. 3

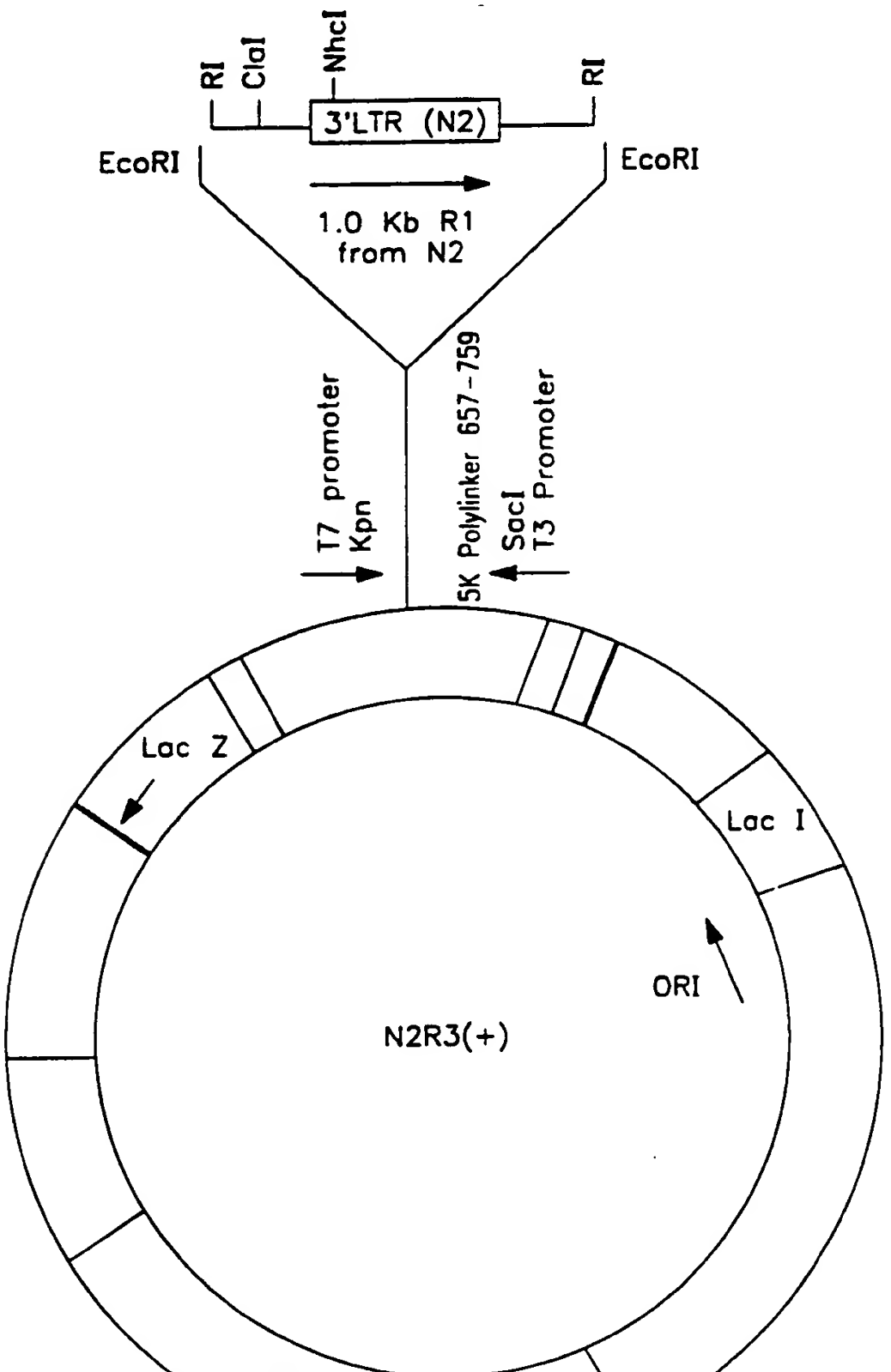

RI
ClaI
NheI
RI
3'LTR (N2)
EcoRI
EcoRI
1.0 Kb R1
from N2
T7 promoter
Kpn
5K Polylinker 657-759
SacI
T3 Promoter
Lac Z
Lac I
ORI
N2R3(+)
Amp

FIG. 4

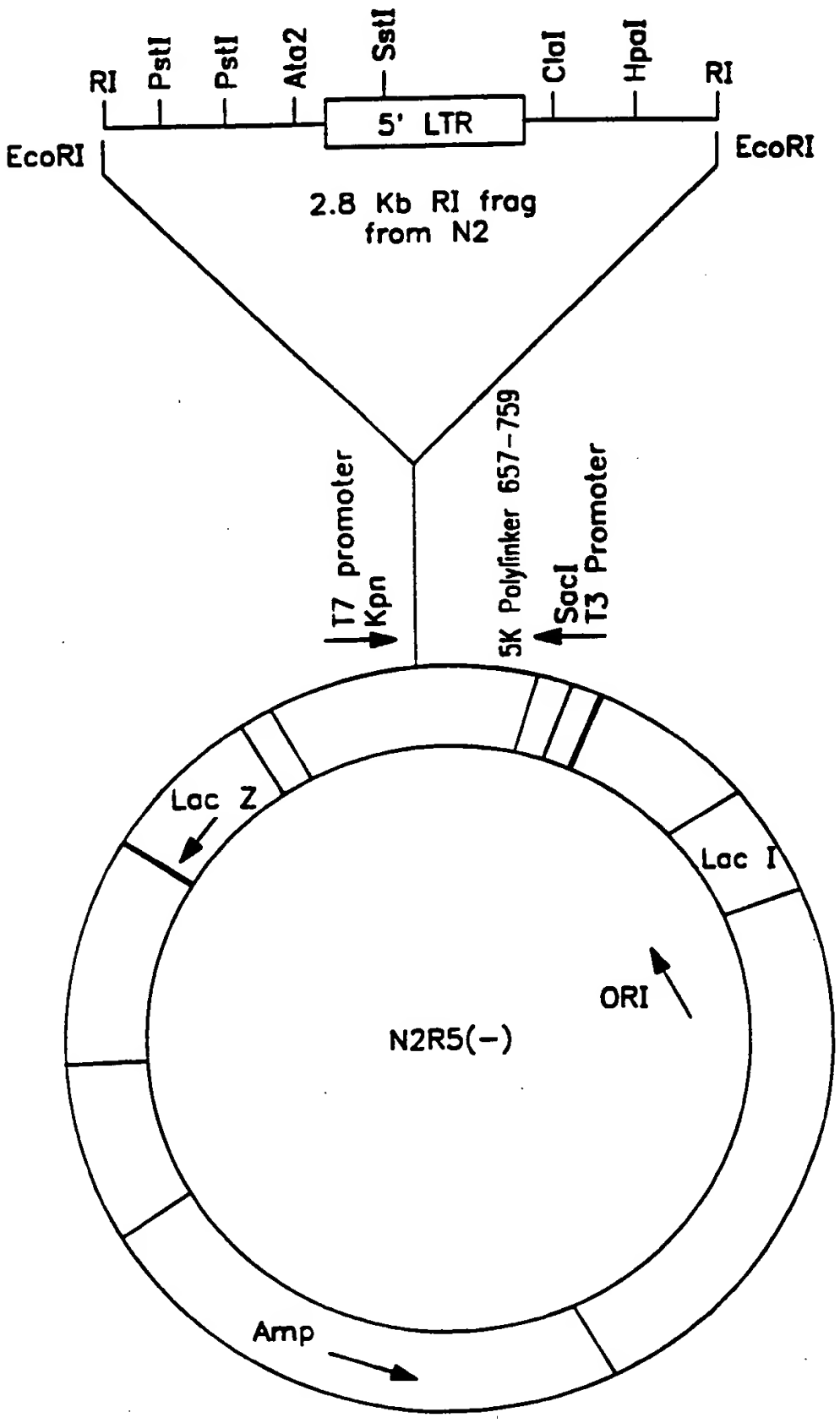

RI
PstI
PstI
Ala2
SstI
ClaI
HpaI
RI
5' LTR
EcoRI
EcoRI
2.8 Kb RI frag
from N2
T7 promoter
Kpn
5K Polylinker 657-759
SacI
T3 Promoter
Lac Z
Lac I
ORI
N2R5(-)
Amp

FIG. 5

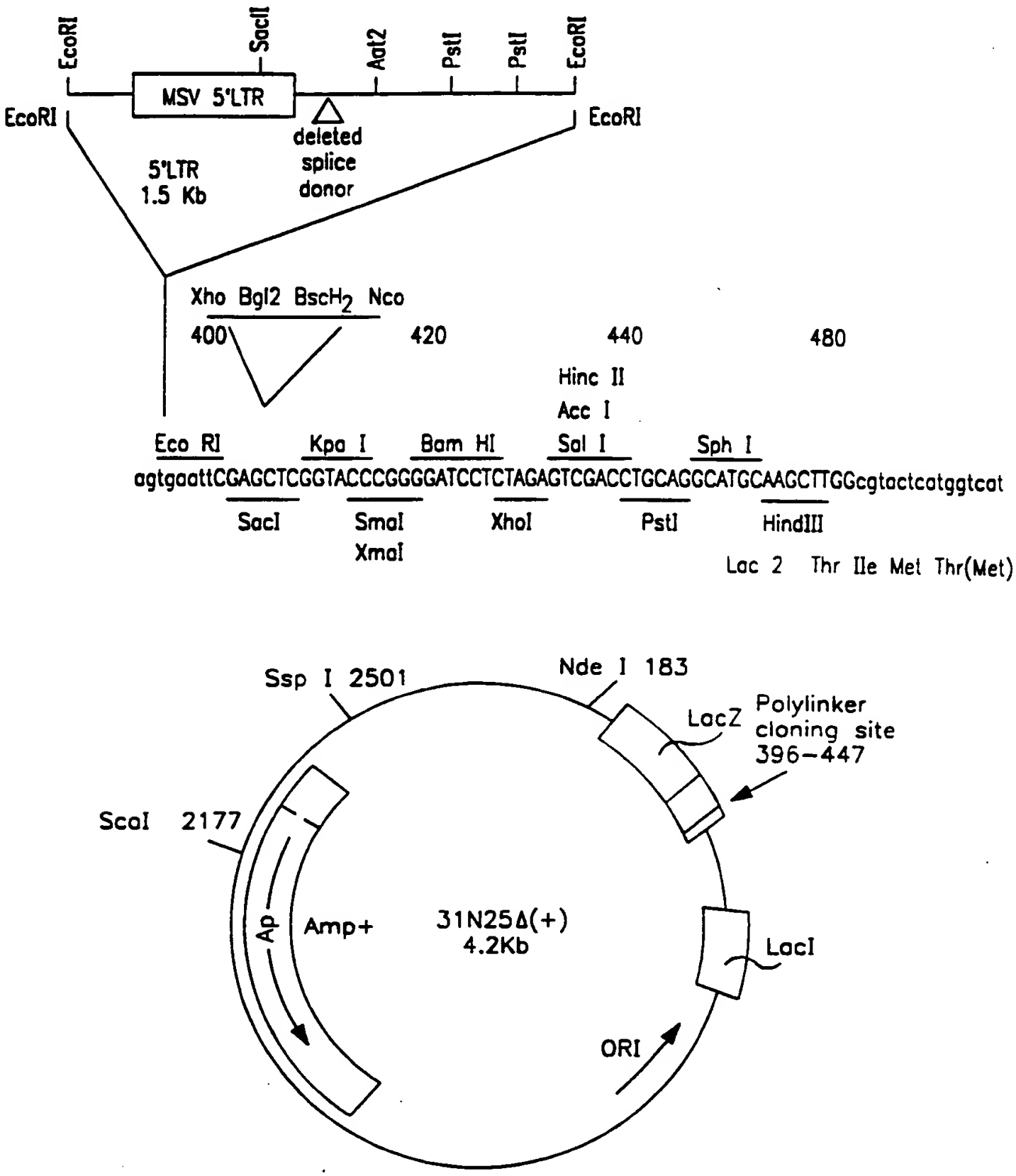

EcoRI
SacII
Aat2
PstI
PstI
EcoRI
MSV 5'LTR
EcoRI
EcoRI
deleted
splice
donor
5'LTR
1.5 Kb
Xho Bgl2 BscH2 Nco
400
420
440
480
Hinc II
Acc I
Eco RI
Kpa I
Bam HI
Sal I
Sph I
agtgaattCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGcgtactcatggtcat
SacI
SmaI
XmaI
XhoI
PstI
HindIII
Lac 2 Thr Ile Met Thr(Met)
Ssp I 2501
Nde I 183
LacZ
Polylinker
cloning site
396–447
ScaI 2177
Ap
Amp+
31N25Δ(+)
4.2Kb
LacI
ORI

FIG. 6

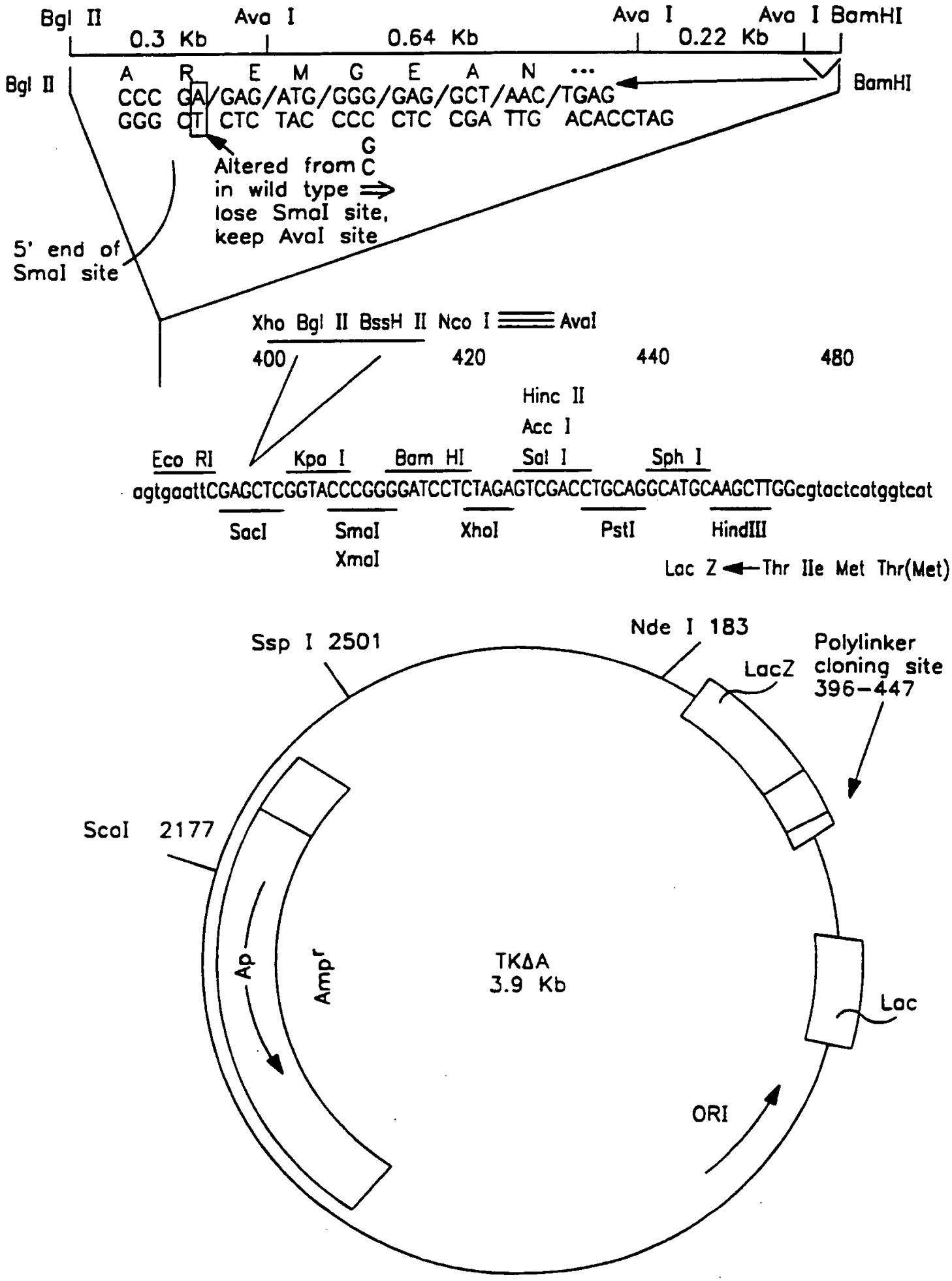

Bgl II
Ava I
Ava I
Ava I BamHI
0.3 Kb
0.64 Kb
0.22 Kb
Bgl II
A R E M G E A N ...
CCC GA/GAG/ATG/GGG/GAG/GCT/AAC/TGAG
GGG CT CTC TAC CCC CTC CGA TTG ACACCTAG
G
Altered from C in wild type ⇒ lose SmaI site, keep AvaI site
BamHI
5' end of SmaI site
Xho Bgl II BssH II Nco I AvaI
400
420
440
480
Hinc II
Acc I
Eco RI
Kpn I
Bam HI
Sal I
Sph I
agtgaattCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGcgtactcatggtcat
SacI
SmaI
XmaI
XhoI
PstI
HindIII
Lac Z ← Thr Ile Met Thr(Met)
Ssp I 2501
Nde I 183
LacZ
Polylinker cloning site 396–447
ScaI 2177
Ap
Ampr
TKΔA
3.9 Kb
Lac
ORI

FIG. 7

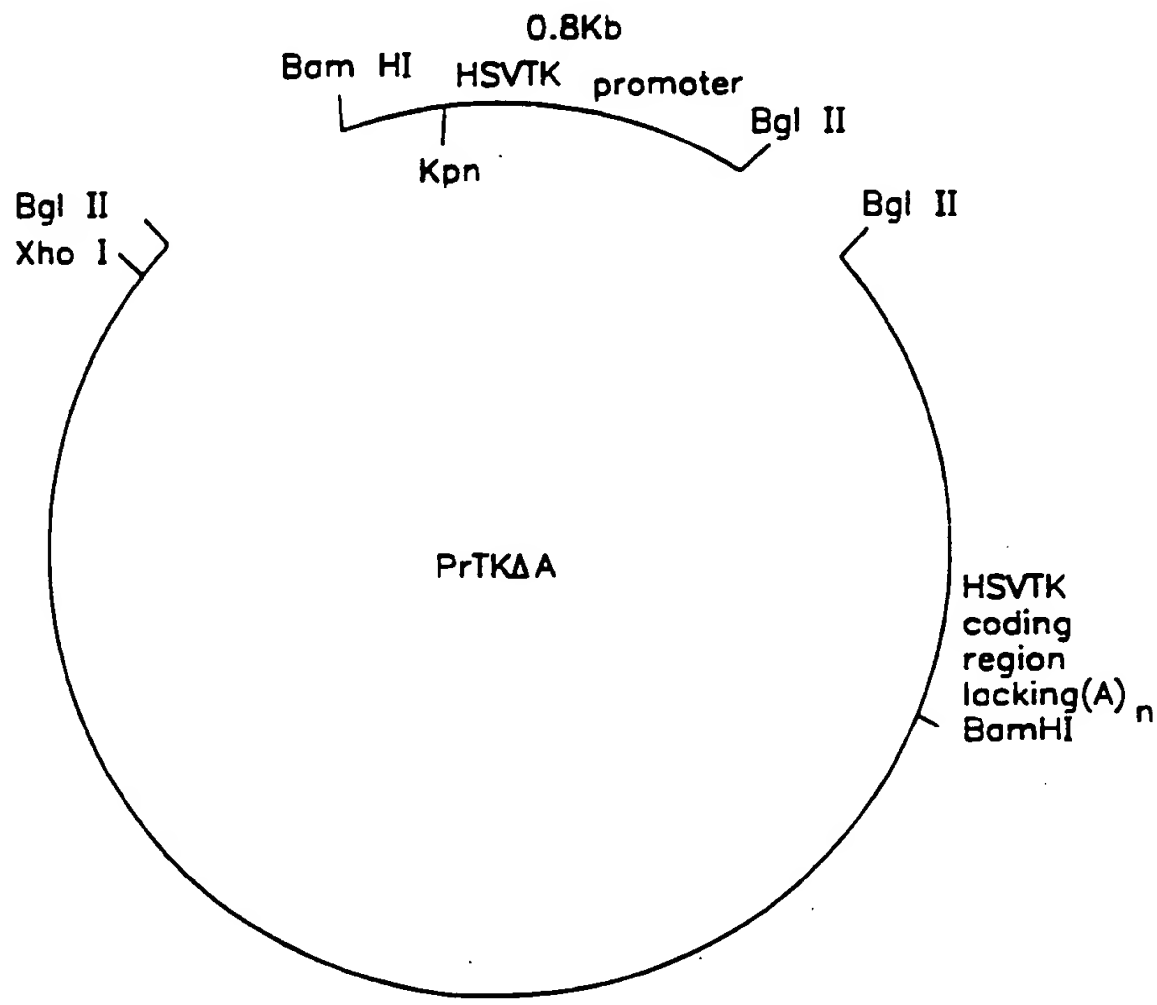

0.8Kb
Bam HI
HSVTK
promoter
Bgl II
Kpn
Bgl II
Xho I
Bgl II
PrTKΔA
HSVTK
coding
region
lacking(A) n
BamHI

FIG. 8

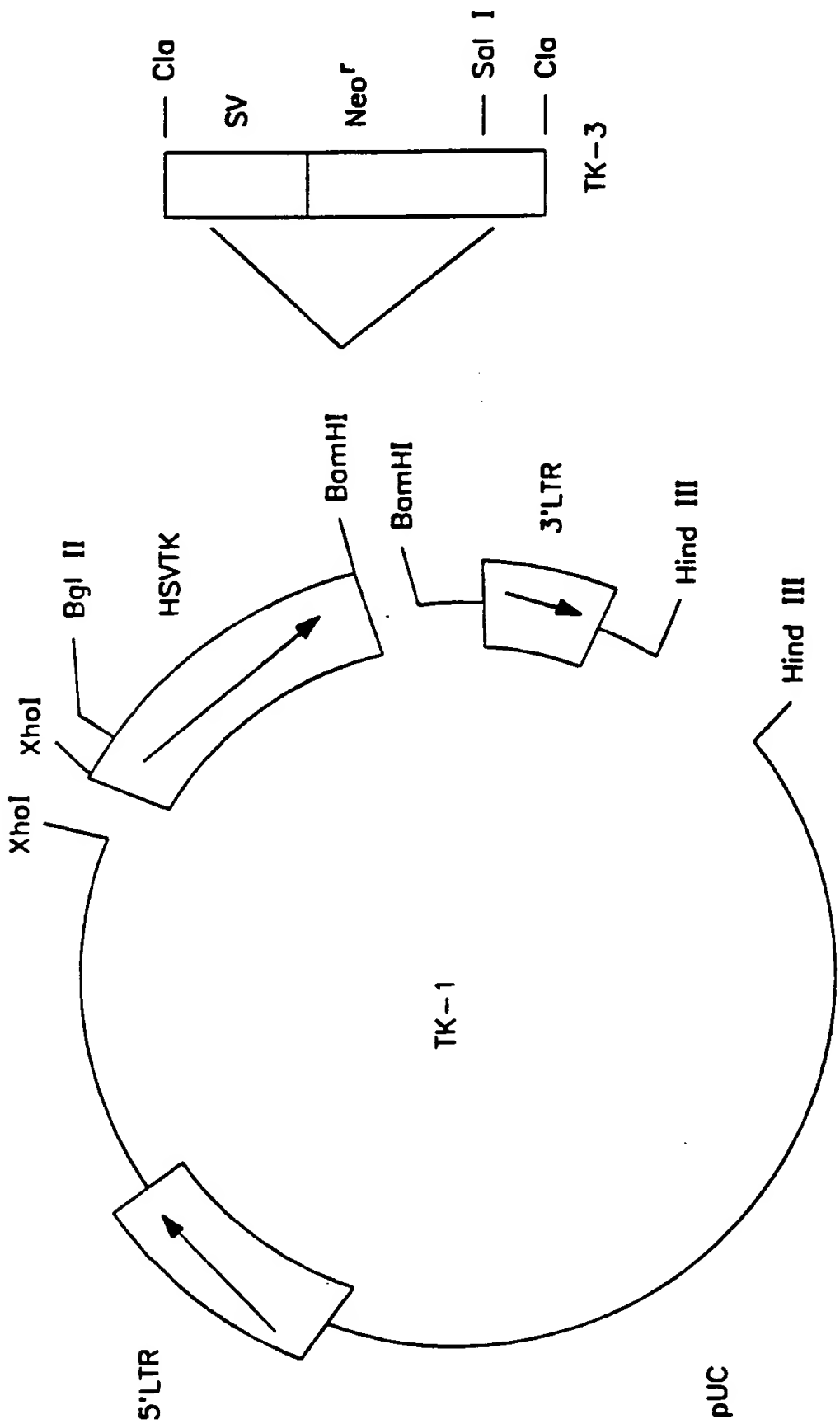

Cla
SV
Neo^r
Sal I
Cla
TK-3
BamHI
BamHI
3'LTR
Hind III
Hind III
HSVTK
Bgl II
XhoI
XhoI
TK-1
5'LTR
pUC

FIG. 9

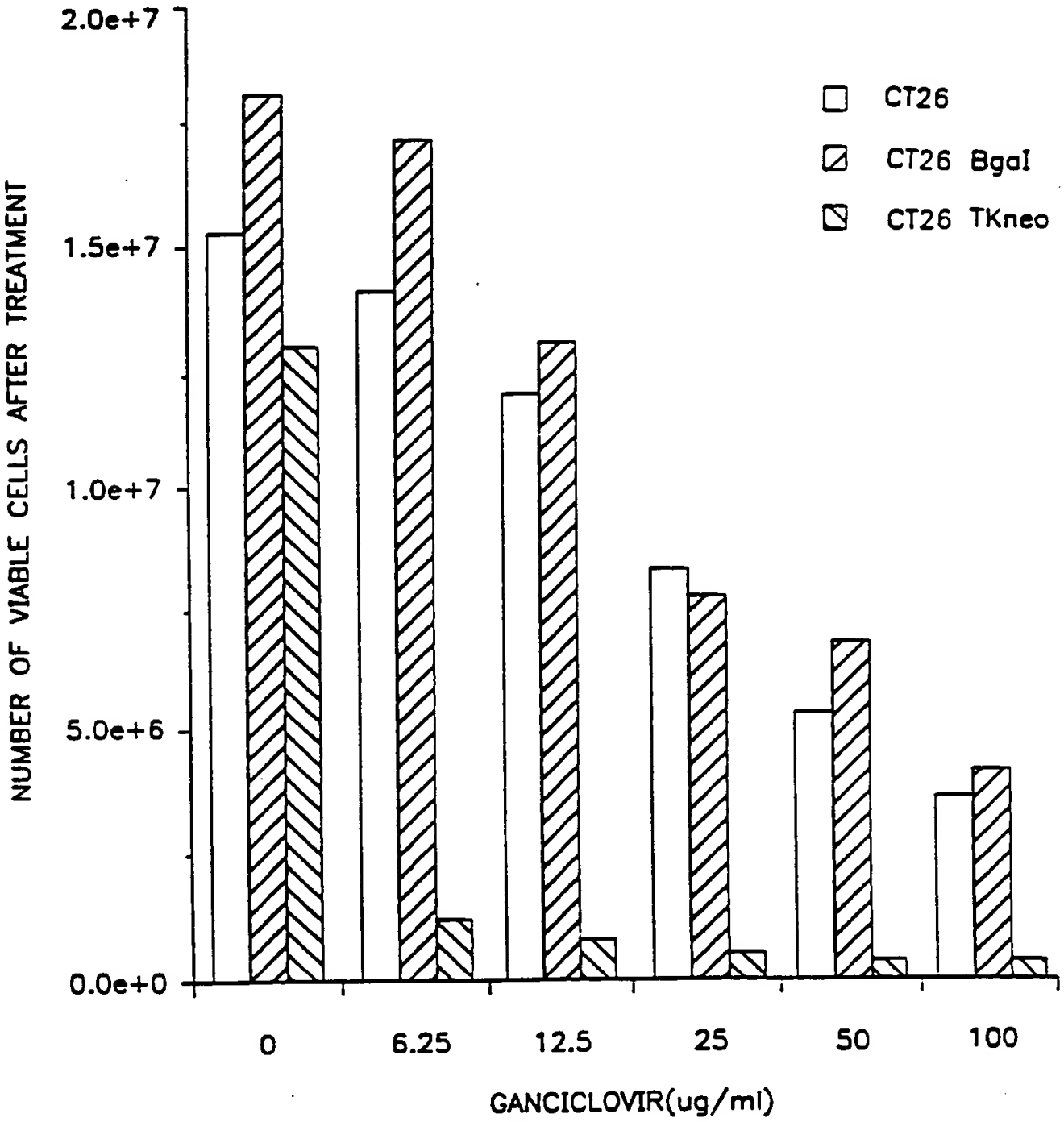

2.0e+7
1.5e+7
1.0e+7
5.0e+6
0.0e+0
NUMBER OF VIABLE CELLS AFTER TREATMENT
0
6.25
12.5
25
50
100
GANCICLOVIR(ug/ml)
CT26
CT26 Bgal
CT26 TKneo

FIG. 10

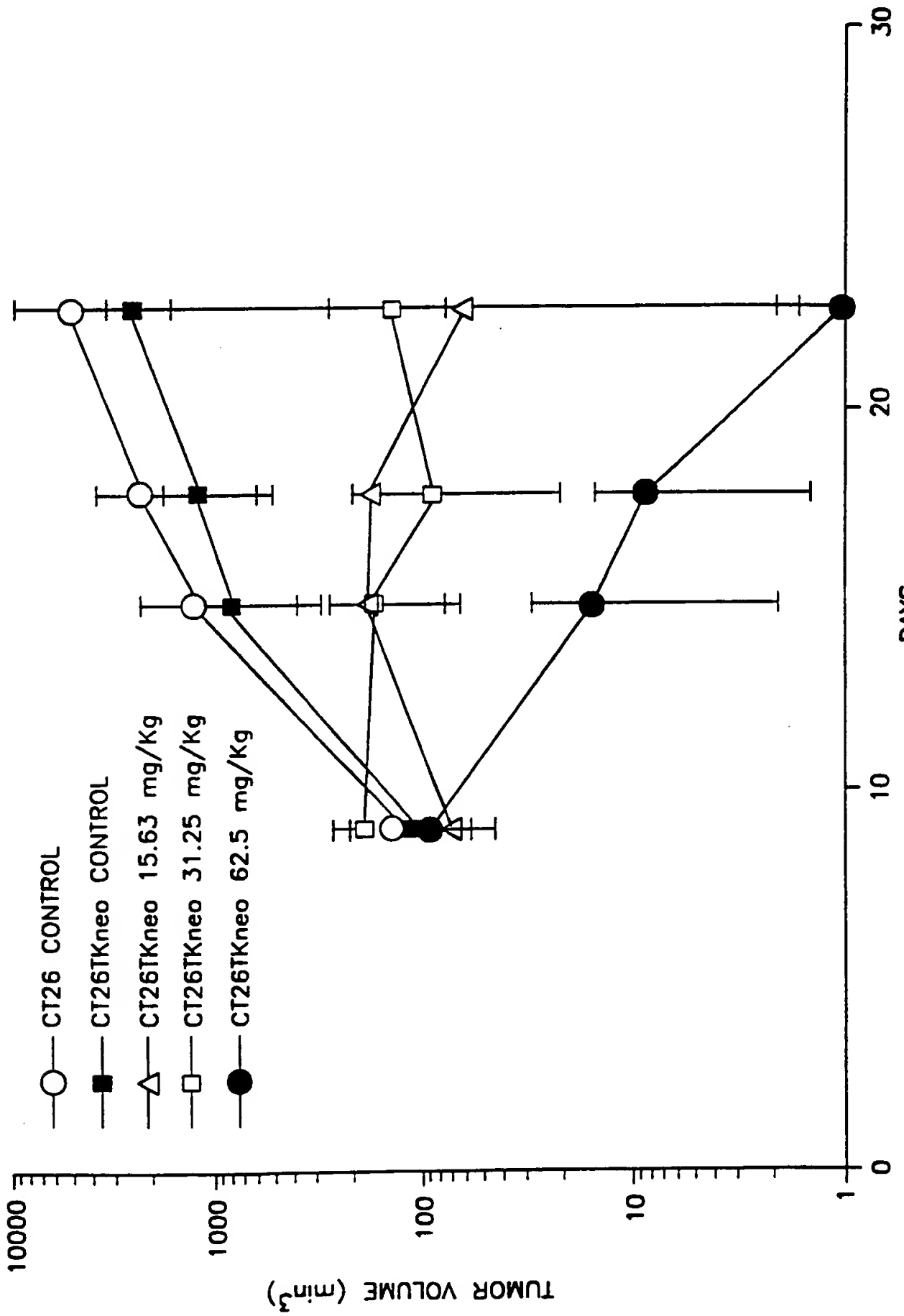
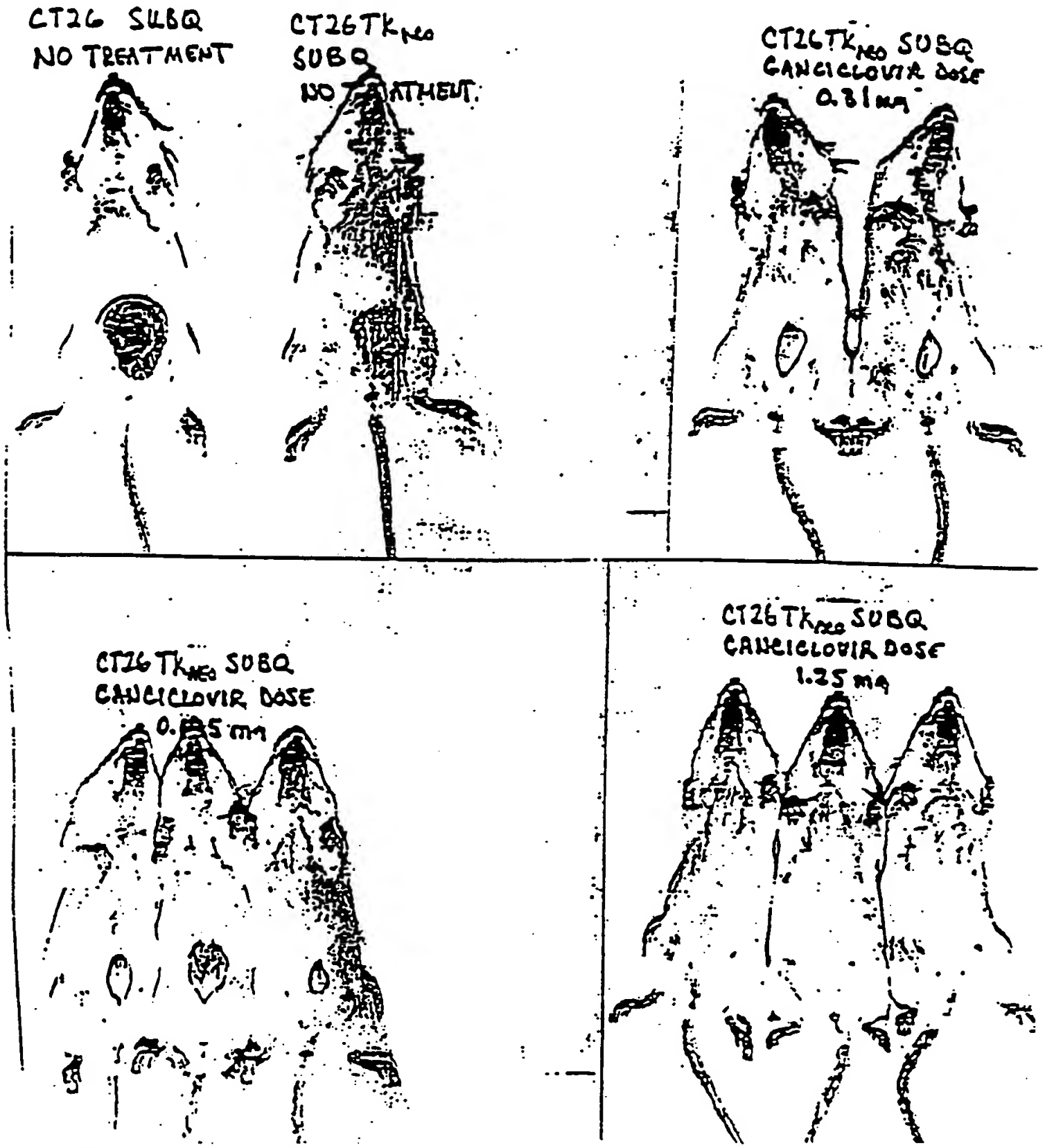

TUMOR VOLUME ($mm^3$)
10000
1000
100
10
1
0
10
20
30
DAYS
CT26 CONTROL
CT26TKneo CONTROL
CT26TKneo 15.63 mg/Kg
CT26TKneo 31.25 mg/Kg
CT26TKneo 62.5 mg/Kg

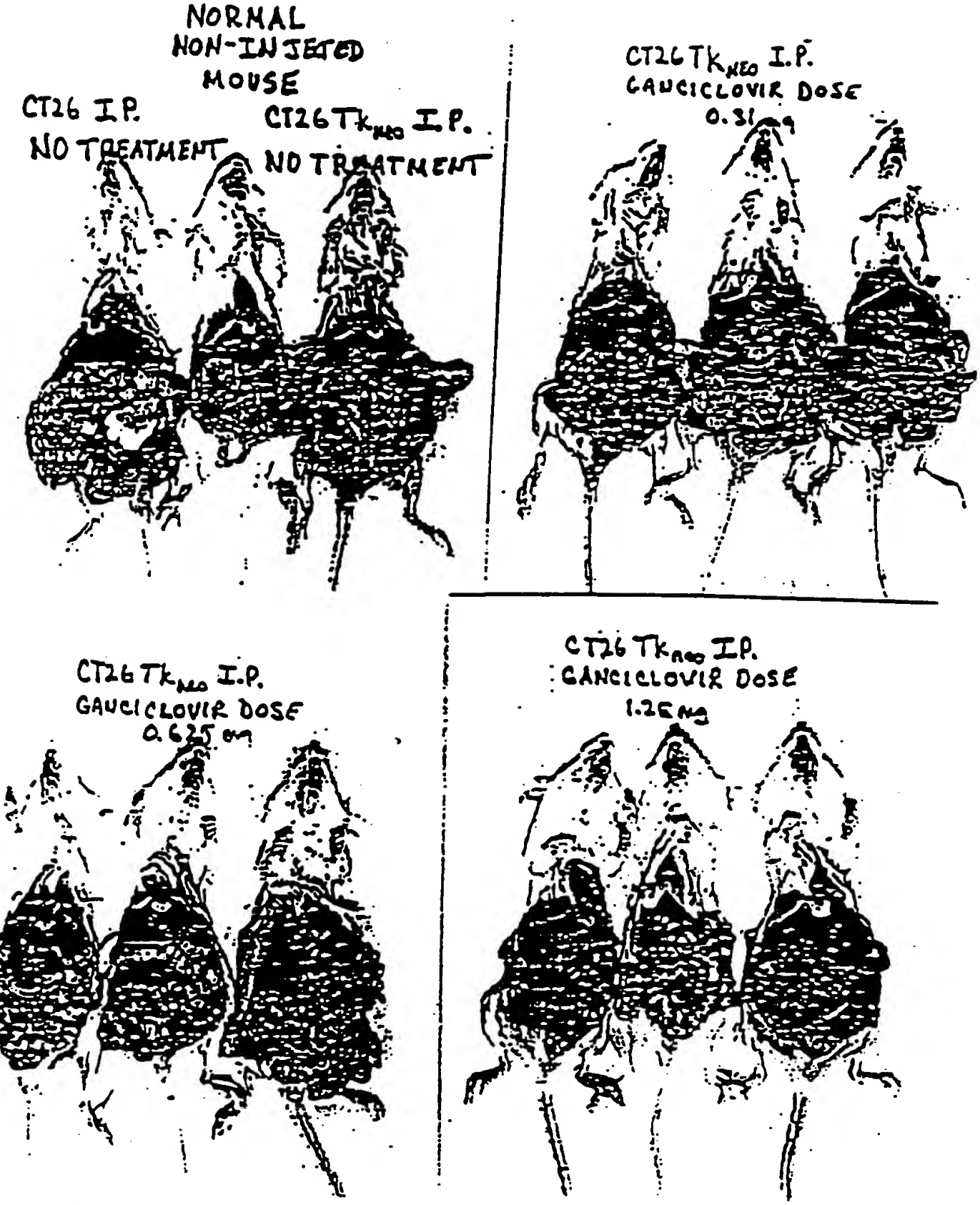
FIG. 11

# FIGURE 12




The Effect of Different Dose Regimen of Ganciclovir on Intraperitoneal CT26TKNeo Tumor Growth

# FIGURE 13




The Effect of Different Dose Regimen of Ganciclovir on Subcutaneous CT26TKNeo Tumor Growth

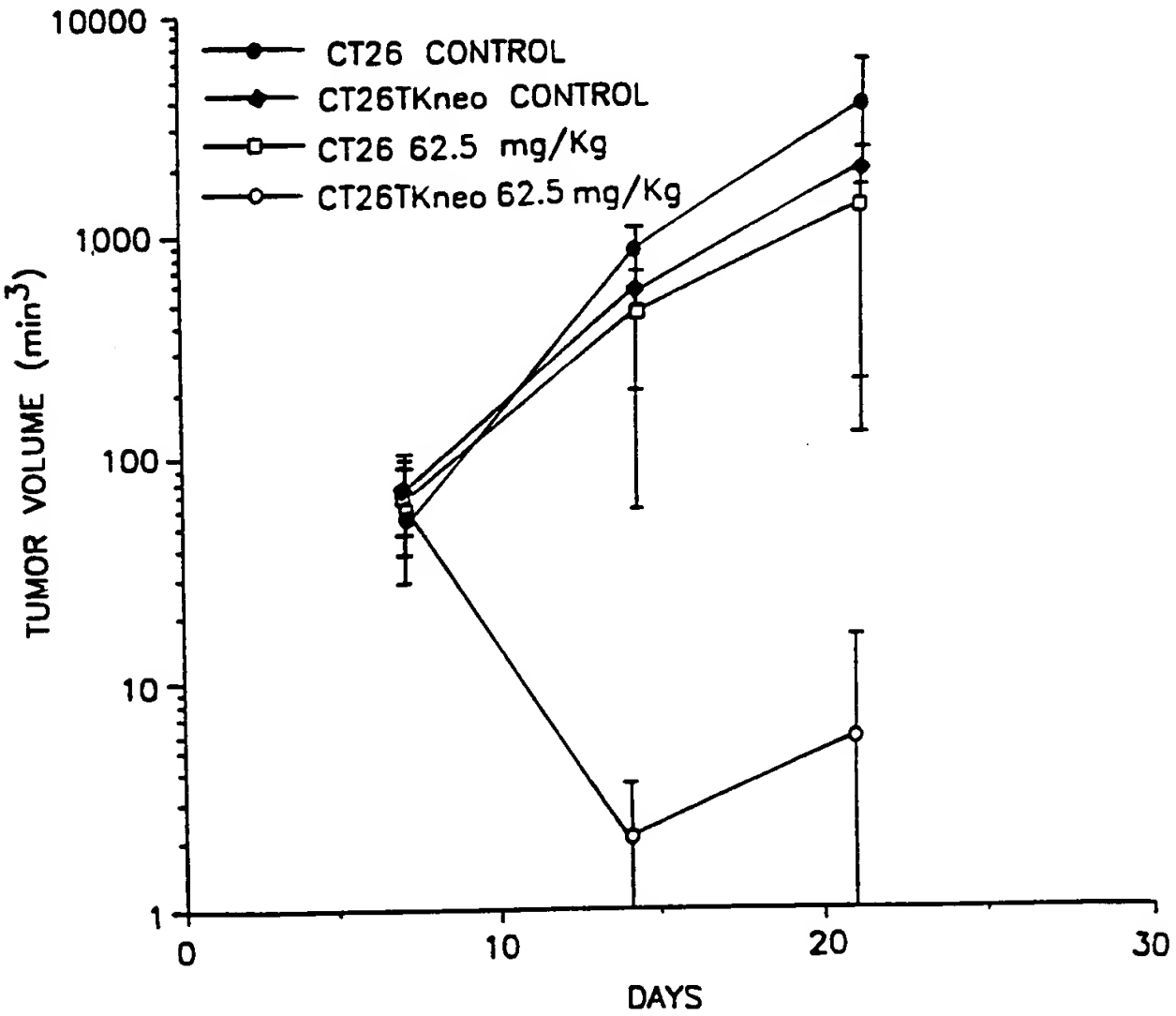
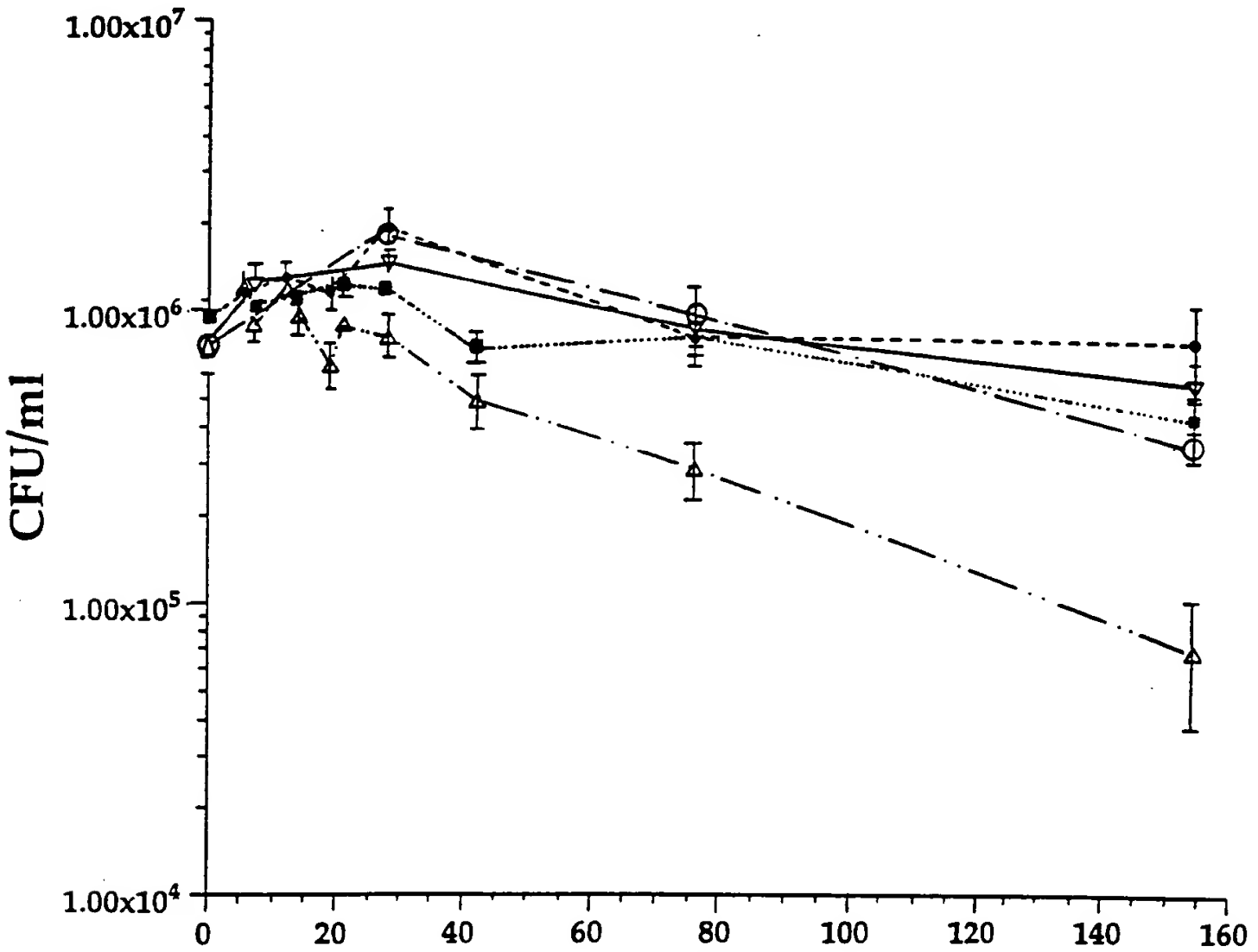

10000
1000
100
10
1
TUMOR VOLUME (min3)
CT26 CONTROL
CT26TKneo CONTROL
CT26 62.5 mg/Kg
CT26TKneo 62.5 mg/Kg
0
10
20
30
DAYS

FIG. 14




**Formulation:**

25mM Tris pH 7.2
60mM NaCl
1 mg/ml Arginine
5 mg/ml HSA
50 mg/ml Lactose

FIG. 15

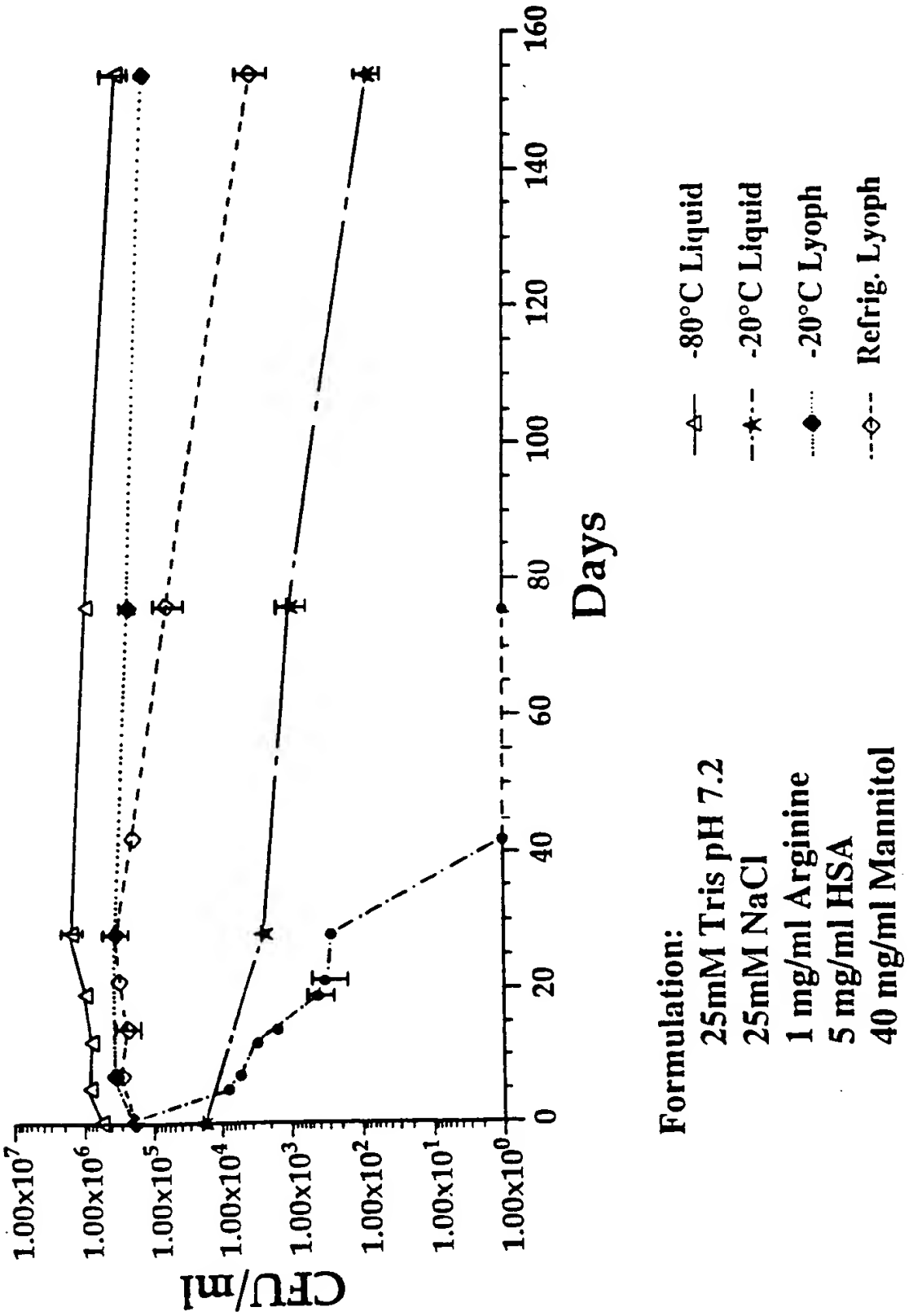
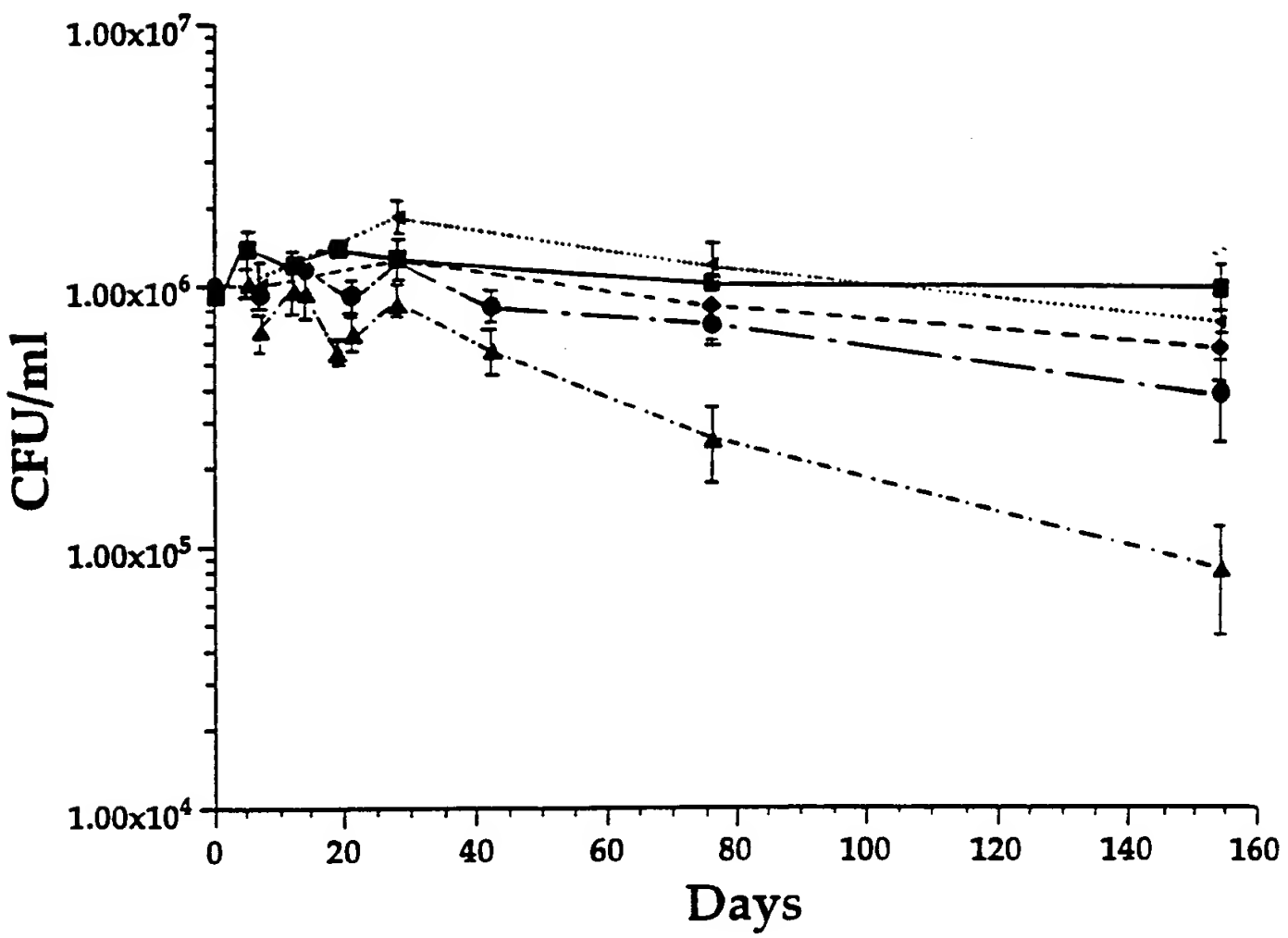

CFU/ml
1.00x10^7
1.00x10^6
1.00x10^5
1.00x10^4
1.00x10^3
1.00x10^2
1.00x10^1
1.00x10^0
0
20
40
60
80
100
120
140
160
Days
-80°C Liquid
-20°C Liquid
-20°C Lyoph
Refrig. Lyoph
R.T. Lyoph
Formulation:
25mM Tris pH 7.2
25mM NaCl
1 mg/ml Arginine
5 mg/ml HSA
40 mg/ml Mannitol

FIG. 16




Formulation:
25mM Tris pH 7.2
60mM NaCl
1mg/ml Arginine
5mg/ml HSA
50mg/ml Trehalose

-80°C Liquid
-20°C Liquid
-20°C Lyoph
Refrig. Lyoph
R.T. Lyoph

FIG. 17

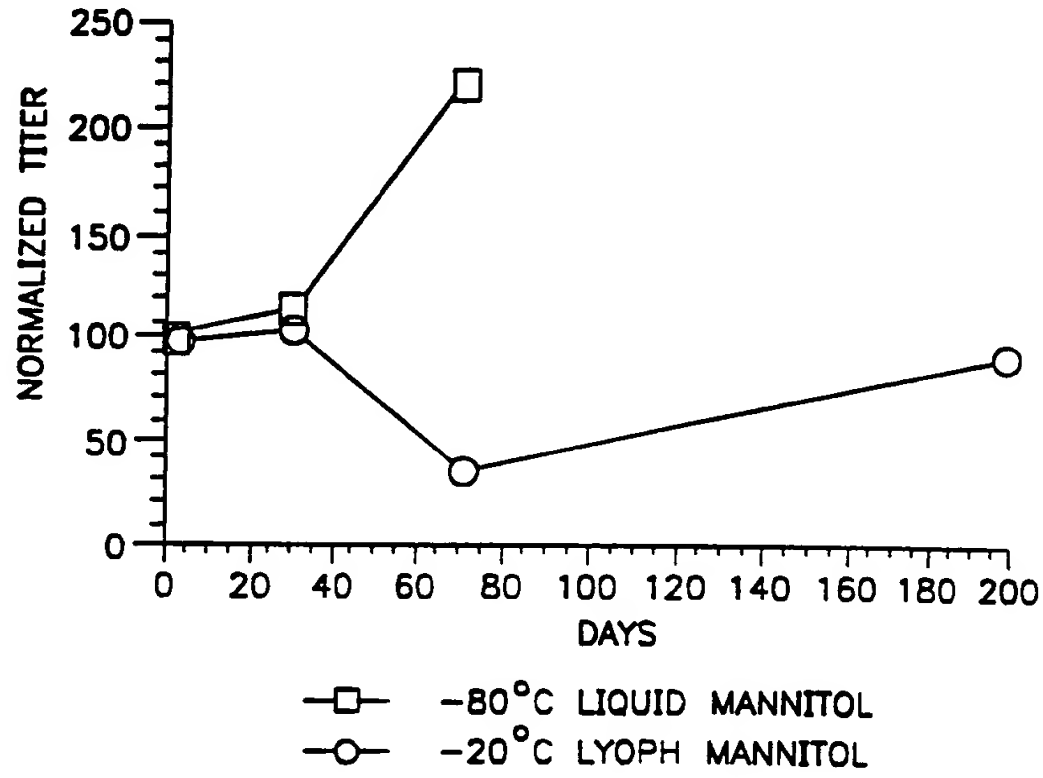



FIG. 18A

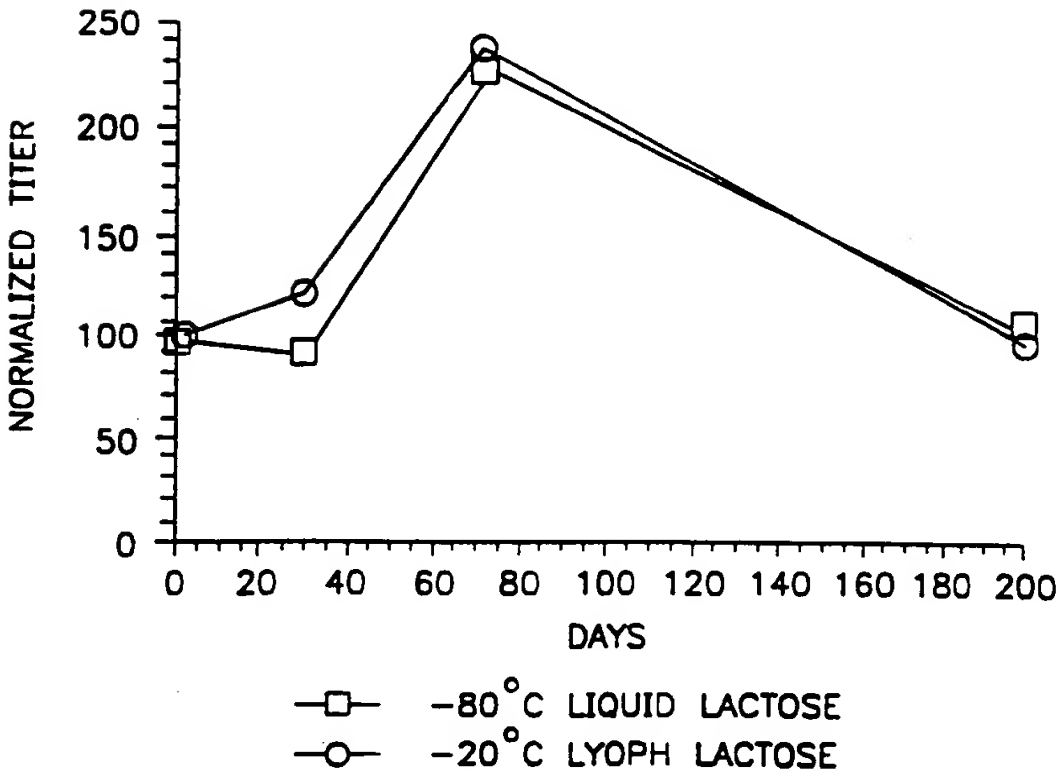



FIG. 18B

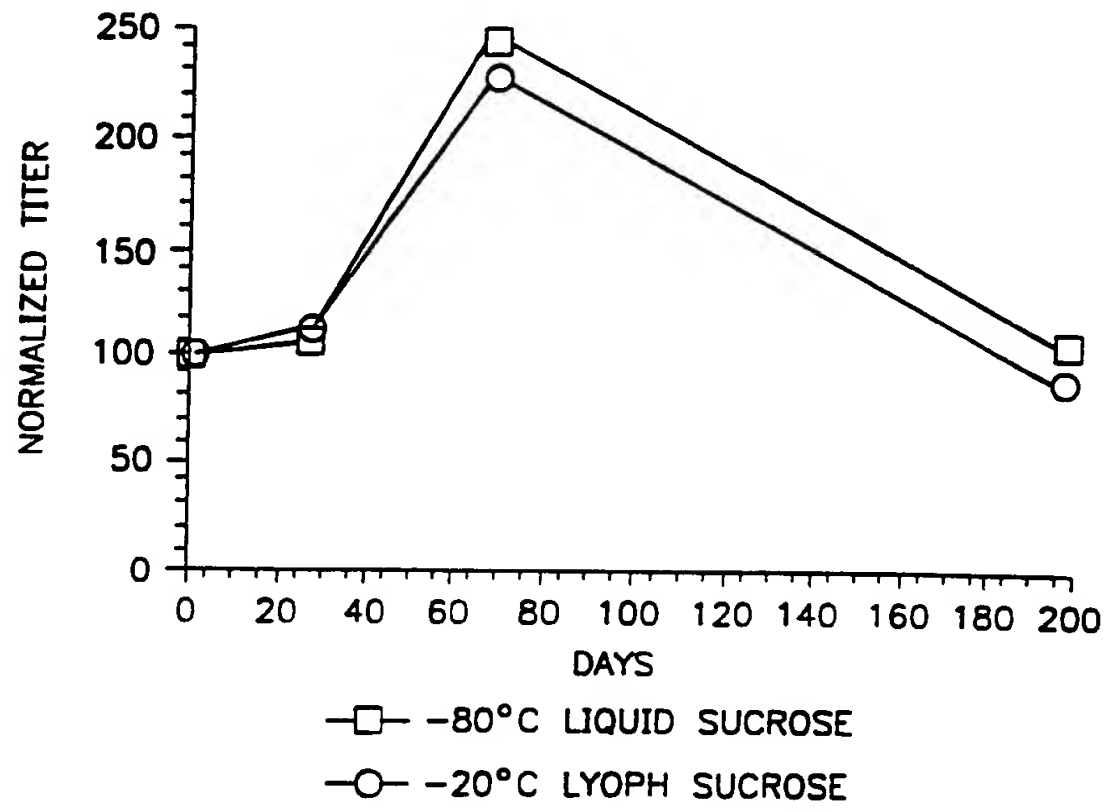



FIG. 18C

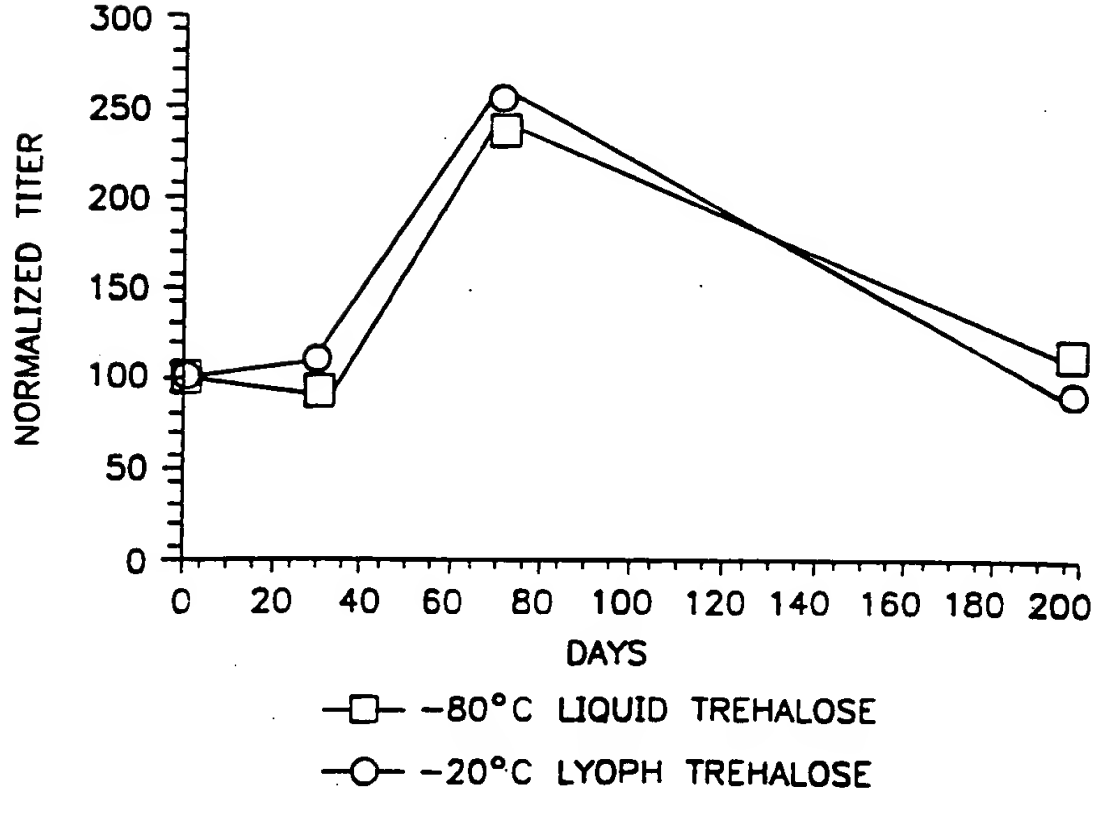



FIG. 18D

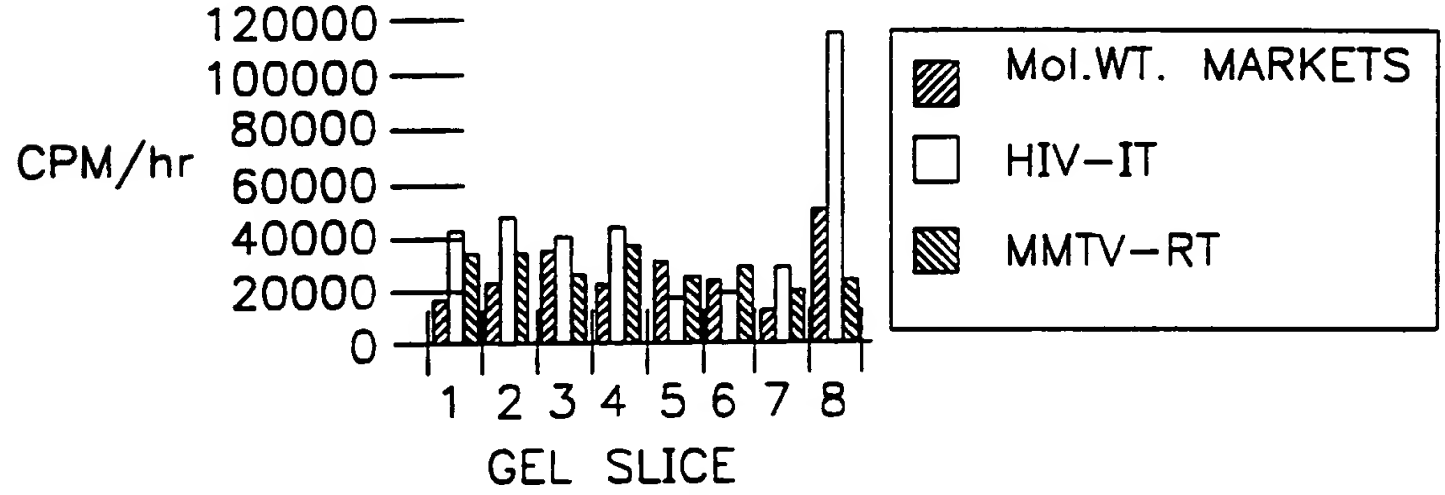

120000
100000
80000
60000
40000
20000
0
CPM/hr
1 2 3 4 5 6 7 8
GEL SLICE
Mol.WT. MARKETS
HIV-IT
MMTV-RT

FIG. 19

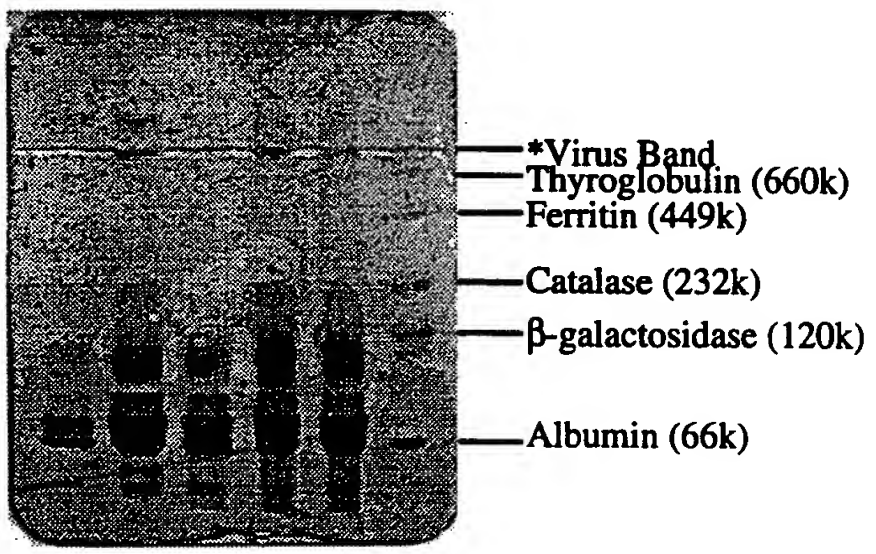

DA/β-gal S500 "nf"
DA/β-gal (8/13) Crude
NB 9314.45 (10/18) Crude
DAC 6A3 (4/28) Crude
HBc 5A2 (5/12) Crude
Markers
*Virus Band
Thyroglobulin (660k)
Ferritin (449k)
Catalase (232k)
β-galactosidase (120k)
Albumin (66k)

FIG. 20

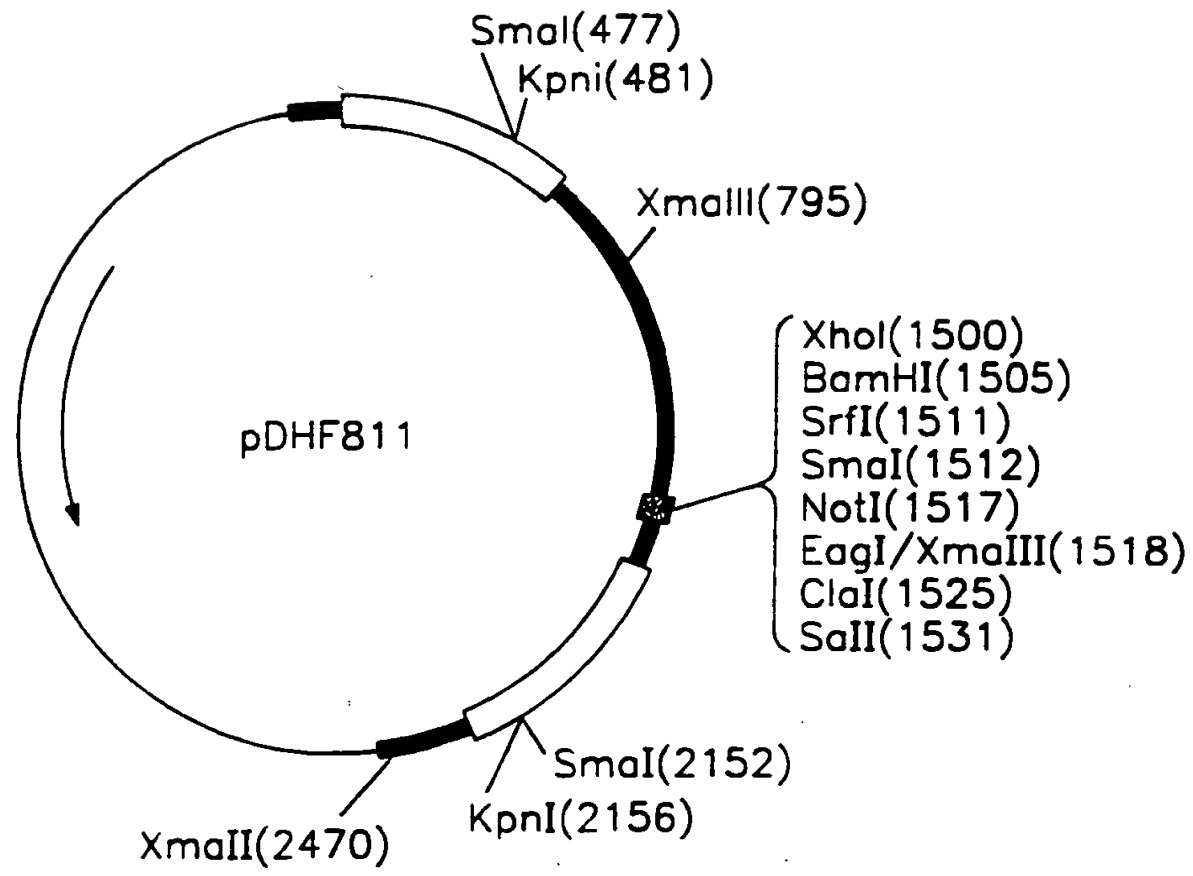

SmaI(477)
KpnI(481)
XmaIII(795)
XhoI(1500)
BamHI(1505)
SrfI(1511)
SmaI(1512)
NotI(1517)
EagI/XmaIII(1518)
ClaI(1525)
SalI(1531)
pDHF811
SmaI(2152)
KpnI(2156)
XmaII(2470)

FIG. 21

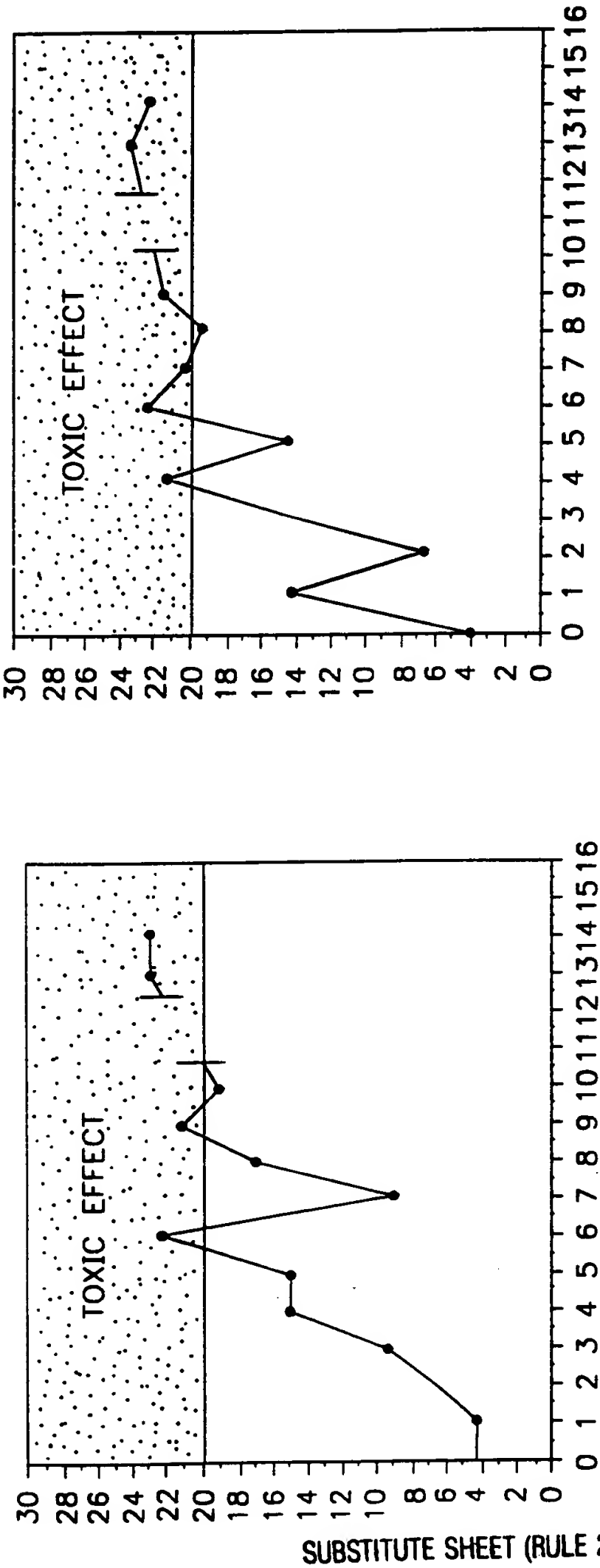



FIG. 22B




FIG. 22A

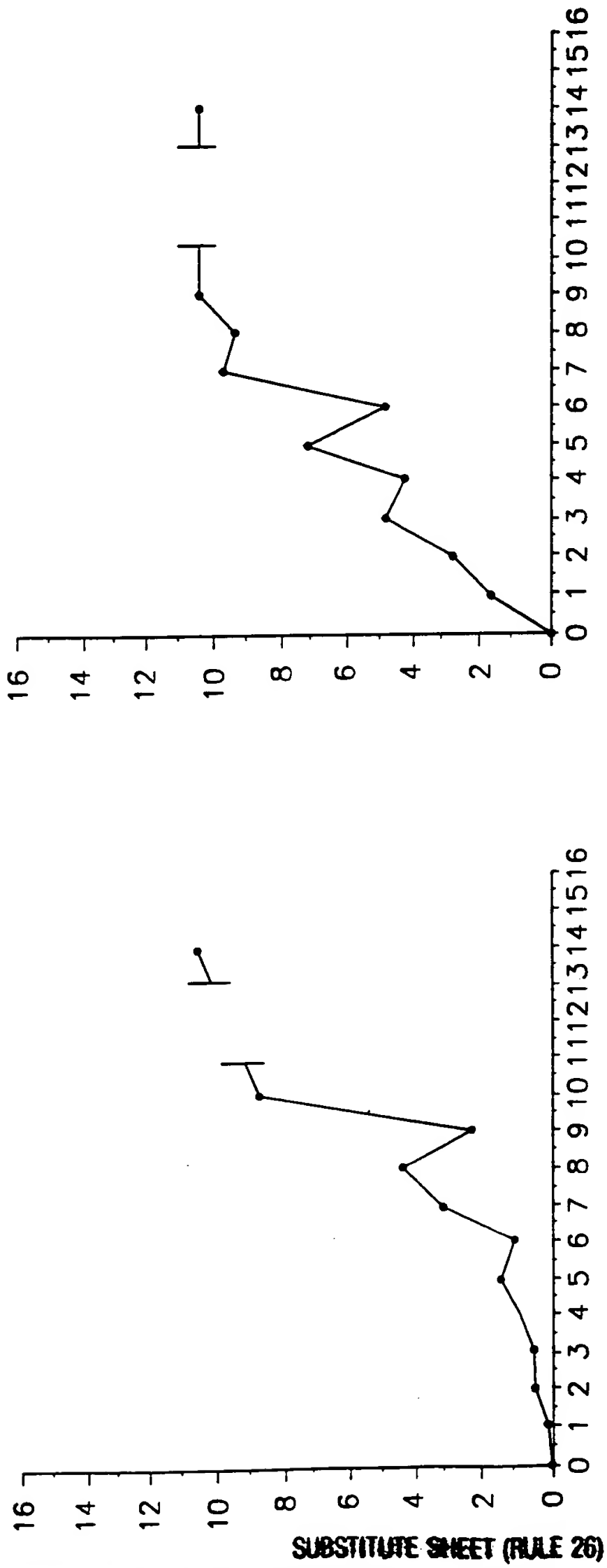



FIG. 22D




FIG. 22C

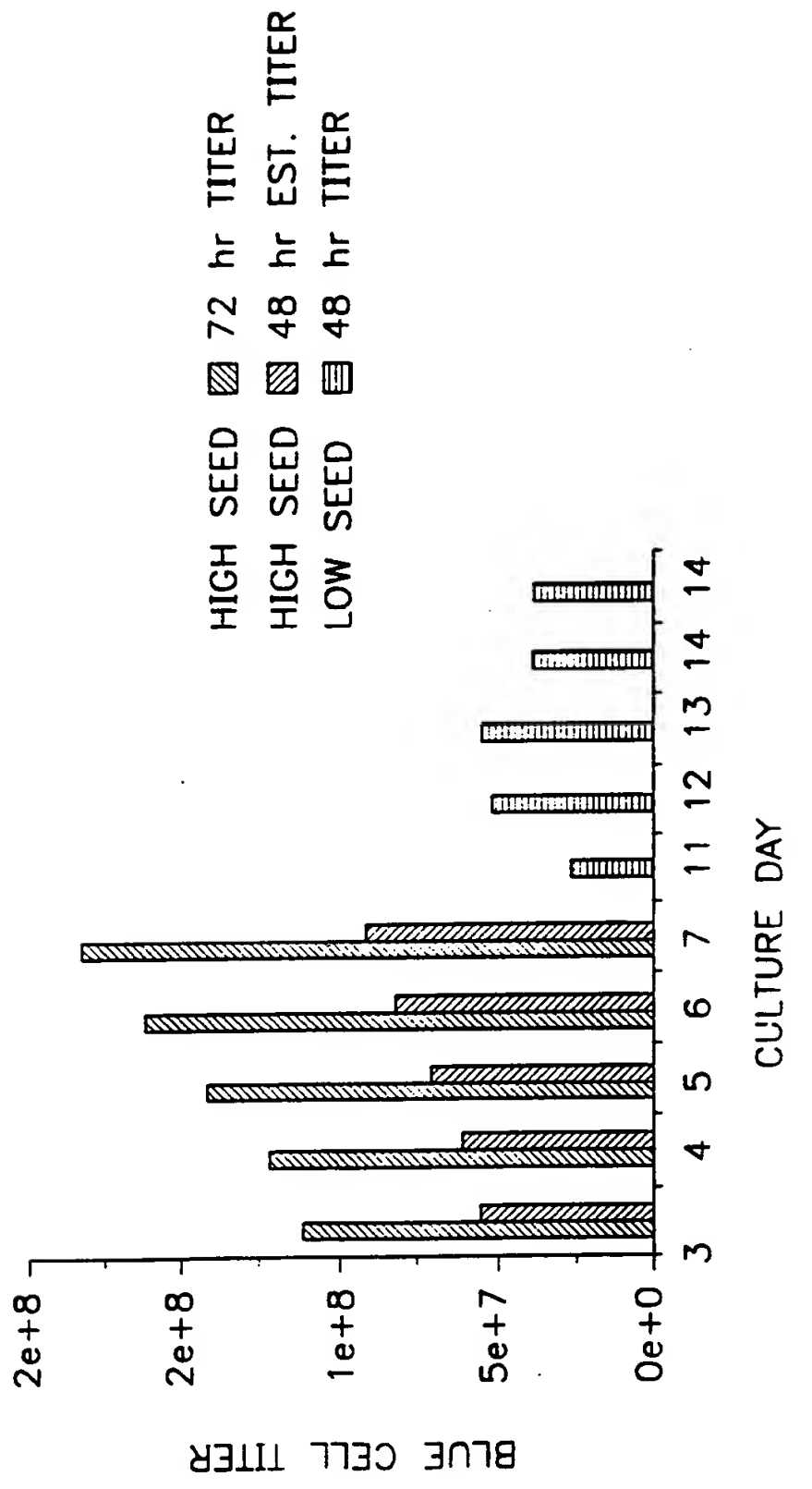

HIGH SEED 72 hr TITER
HIGH SEED 48 hr EST. TITER
LOW SEED 48 hr TITER
BLUE CELL TITER
2e+8
2e+8
1e+8
5e+7
0e+0
3
4
5
6
7
11
12
13
14
14
CULTURE DAY

FIG. 23